THE ANGIOGENIC RESPONSE TO BRADYKININ *IN VITRO*

The role of Bradykinin receptors in hypoxic hearts and tumors

Inauguraldissertation

zur Erlangung der Würde eines Doktors der Philosophie
vorgelegt der Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
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Aus Teheran, Iran und Lörrach, Deutschland

Basel 2004

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

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Basel, den 29. Juni 2004

Prof. Dr. Marcel Tanner
Dekan der Philosophisch-Naturwissenschaftlichen Fakultät

To Mitra, Radjab, Nima and Mona Neysari

ACKNOWLEGMENTS

I am most thankful to Professor Edouard Battegay for giving me the opportunity to perform my studies in his laboratory. He always challenged me to work creatively and enabled me to develop my scientific and intellectual skills. I am thankful to Professor Theodor W. Güntert, who accepted to be the chairman of my PhD committee and who kept on supporting me all along. I would like to thank him also for his critical review of my work and the many positive suggestions, which he contributed. I am grateful to Professor Karl G. Hofbauer, who accepted to be a member of my PhD committee and for critically reading my manuscript.

I am grateful to Dr. Rok Humar who guided me beyond his responsibility; his professional advice and human understanding were instrumental in my completion of this task. I would like to thank Dr. Fabrice Kiefer for the pleasant time we had in the lab and Mrs. Kaija Paris for taking care of so many things. I would like to thank Dr. Carl-Alexander Scheef for his unconditional help through all the difficult times. I am thankful to Mr. Esfandiar Ardalan who never stopped believing in me, supporting me and encouraging me. I am thankful to Mrs. Laurence Hilfiger for always being there for me. My thoughts are with Mrs. Magda Scheef who has always inspired me.

No words can express how thankful I am to my parents, who trusted me, allowing me to achieve what I have; their love and support has been my sustenance for the past 15 years. They have always been willing to sacrifice themselves for their children. I hope my efforts will repay them in some small way for all of theirs.

Last but not least I would like to thank Mr. Bardia Kalantari, who came into my life at its most difficult time, and whose love has helped me to carry on.

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SUMMARY

End organ damage resulting from hypertension is a leading cause of morbidity and mortality worldwide. In hypertension, left ventricular mass increases resulting in left ventricular hypertrophy (LVH). LVH increases the risk of heart failure and sudden cardiac death. This is due to the decreased supply of oxygen and nutrients (ischemia) to the myocardium because of vascular rarefaction. Research has focused on inducers of angiogenesis such as basic fibroblast growth factor and vascular endothelial growth factor to improve myocardial oxygenation and function. However, recently components of the Renin-Angiotensin-Aldosteron System (RAAS), which contributes to blood pressure control, have been shown to affect angiogenesis. Angiotensin-converting-enzyme (ACE) inhibitors are used to treat high blood pressure and congestive heart failure. These block the conversion of physiologically inactive angiotensin I to active vasoconstrictive angiotensin II and inhibit the breakdown of Bradykinin (BK), a potent vasodilator and mediator of inflammation. ACE inhibitors increased capillary density in ischemic tissue by the induction of new microvessels in ischemic rat limbs *in vivo*. Several lines of evidence suggest Bradykinin to possess significant angiogenic activity. Hence, Bradykinin may mediate the effect of ACE inhibitors. Still, it is unclear through whether Bradykinin promotes vascularization of the ischemic heart via the Bradykinin receptor subtype 1 or 2. On the other hand, blocking angiogenesis could be a strategy to arrest tumor growth, since tumor growth and metastasis depend on angiogenesis. However, it is yet to be fully elucidated whether and through which mechanisms Bradykinin induces angiogenesis in tumors.

Therefore, the aim of this thesis was in the first line to clarify the angiogenic potential of Bradykinin in the ischemic heart *in vitro*, especially the roles of the two Bradykinin receptor subtypes in the regulation of Bradykinin-induced angiogenesis. In second line, the thesis aims to comparatively assess the role of Bradykinin and requirement of Bradykinin receptors in cancer, i.e. melanomas.

To do so, we used an *in vitro* model of angiogenesis of the murine heart under moderate hypoxic conditions (3% O_2). Pilot experiments showed decreased angiogenic potential of hypertrophied rodent hearts compared to normal healthy controls. When using ACE inhibitors, angiogenesis *in vitro* of hypoxic normal and hypertrophied hearts increased, and, interestingly, Bradykinin showed a potent induction of capillary like sprout formation.

This angiogenic effect was induced at low (10nM) but not at high concentrations of Bradykinin (1mM). RT-PCR showed expression of both Bradykinin receptor subtypes in hypoxic mouse hearts. The angiogenic response to Bradykinin was inhibited by a specific Bradykinin receptor 2 (BKR2) inhibitor, but not by an inhibitor of Bradykinin receptor 1 (BKR1). A specific BKR1 agonist reduced angiogenesis. Bradykinin-induced angiogenesis was not impaired in BKR1 (-/-) mouse hearts. Different nitric oxide synthase inhibitors (L-NAME, L-NIL, NIO) almost completely abrogated the *in vitro* mouse heart angiogenesis response to Bradykinin. Bradykinin did not induce angiogenesis in hearts of iNOS (-/-) mice. Thus, in mouse hearts *in vitro* Bradykinin at low nanomolar concentrations is angiogenic under conditions of prolonged hypoxia. This angiogenic effect is mediated by BKR2 activation and depends on iNOS.

To assess the involvement of Bradykinin in cancer angiogenesis, melanomas were injected and grown in the ear of wildtype and BKR1 (-/-) mice, which acquired a BKR1 (-/-) phenotype vasculature. In contrast to the findings in hearts, we found that in melanomas from BKR1 (-/-) mice angiogenesis *in vitro* was significantly lower as compared to wildtype control. This suggests that melanomas in contrast to hearts require vasculature with functional BKR1 to develop new microvessels.

In summary the key findings of this thesis are the following: Bradykinin potently induces angiogenesis *in vitro* of the hypoxic heart at nanomolar concentrations via BKR2. At high Bradykinin concentrations or using specific BKR1 agonists the angiogenic effect appears to be blocked. Furthermore, functional iNOS is required for Bradykinin to induce angiogenesis *in vitro* of the heart. In contrast to the heart endothelial sprouting and angiogenesis, hypoxic melanomas *in vitro* require BKR1.

Thus, specific stimulation of the BKR2 of the heart vasculature may be a target to reduce tissue ischemia by angiogenesis in the ischemic and/or hypertrophied heart.

ABBREVIATIONS

ACE	angiotensin converting enzyme
Ang II	angiotensin II
bFGF	basic fibroblast growth factor
BK	Bradykinin
BKR1	Bradykinin receptor 1 subtype
BKR2	Bradykinin receptor 1 subtype
CLS	capillary-like sprout formation
EC	endothelial cells
eNOS	endothelial nitric oxide synthase
HIF-1	hypoxia-inducible factor 1
iNOS	inducible nitric oxide synthase
KKS	kallikrein kinin system
L-NAME	nitro-L-arginine methyl ester
L-NIL	L–N 6 –(1–Iminoethyl)lysine, 2Hcl
LVH	left ventricular hypertrophy
MAPK	mitogen activated protein kinase
mTOR	mammalian target of rapamycin
NFk-B	nuclear factor k-B
NIO	N-iminoethyl-L-ornithine
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
PDGF-BB	platelet-derived growth factor B-chain homodimer
RAAS	renin angiotensin aldosteron system
RT-PCR	reverse transcription polymerase chain reaction
SHR	spontaneously hypertensive rats
SMC	smooth muscle cells
VEGF	vascular endothelial growth factor

1. INTRODUCTION

1.1. Formation of new blood vessels

1.1.1. Vasculogenesis, angiogenesis and arteriogenesis

Formation of new blood vessels involves three fundamentally different processes: vasculogenesis, angiogenesis, and arteriogenesis (Figure 1). In vasculogenesis, blood vessels form de novo during embryogenesis. Vasculogenesis consists of the differentiation of angioblasts (the precursors of endothelial cells) into blood islands, which then fuse to form primitive capillary plexuses [1,2].

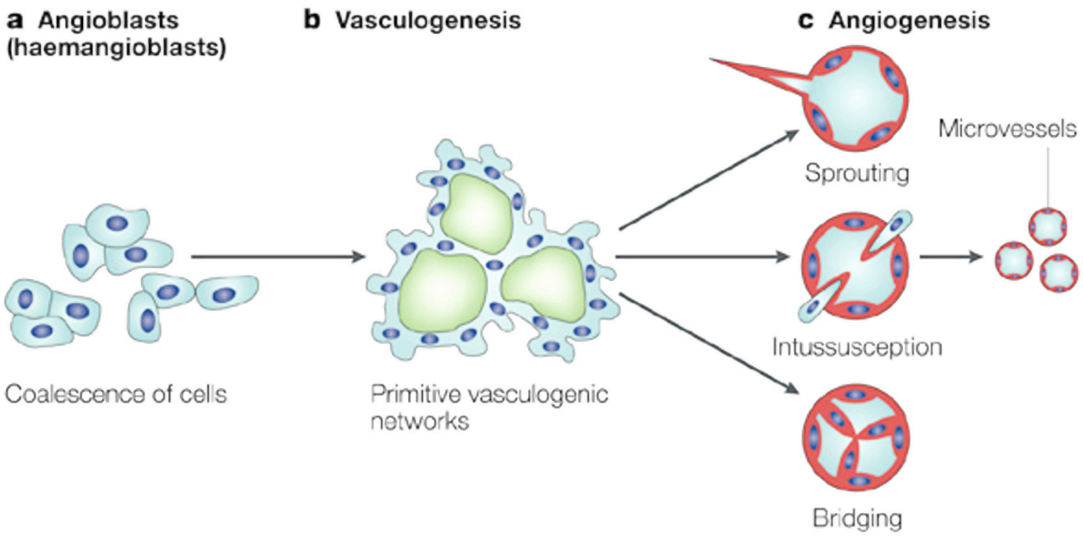


Figure 1. Schematic overview of vasculogenesis and angiogenesis, showing how endothelial-cell precursors (angioblasts and haemangioblasts) coalesce and differentiate into endothelial cells (a), and form primitive vasculogenic networks (vasculogenesis) (b). Remodeling of these networks occurs through angiogenesis (c), which involves sprouting, intussusceptions and/or bridging, resulting in the formation of microvessels. Figure by Bergers et al. [137].

Angiogenesis refers to the formation of new blood vessels from existing micorvessles. These new vessles subsequently grow by sprouting and tube formation to invade later target tissues. It serves the supply of oxygen, nutrients, and the removal of waste [3,4]. During subsequent arteriogenesis, which is defined by rapid proliferation of pre-existing collateral arteries, vessels re-assemble and develop a multilayered muscular coat, which provides blood vessels with viscoelastic and

vasomotor properties. Arteriogenesis is a process that is both phenomenologically and mechanistically totally different from angiogenesis [1,5-8] (Figure 1).

1.1.2. Angiogenesis

Angiogenesis is the formation of new capillary blood vessels from existent microvessels by sprouting, i.e., cellular elongation and outgrowth. Angiogenesis occurs during development, wound healing, ischemic heart disease, ischemic peripheral vascular disease, tumor growth and tumor metastazation [9,10]. Angiogenesis is thought to involve a series of events including (see also Figure 2): 1) activation of endothelial cells within a pre-existing vessel and vasodilation of the parent vessel mediated by NO; 2) degradation of the basement membrane and extracellular matrix; 3) migration of activated endothelial cells from the parent vessel towards the site where angiogenesis is required and where angiogenic inducers are expressed and secreted; 4) proliferation of endothelial cells in the newly forming vessels; 5) re-differentiation of these endothelial cells and recruitment of pericytes along the newly formed vascular structures; 6) formation of a new basement membrane; and finally 7) remodeling of the neovascular network, with maturation and stabilization of the blood vessels [11,12]. Each of these steps is highly regulated by an extensive number of different interacting intracellular and extracellular molecules and cellular receptors [6,7,9].

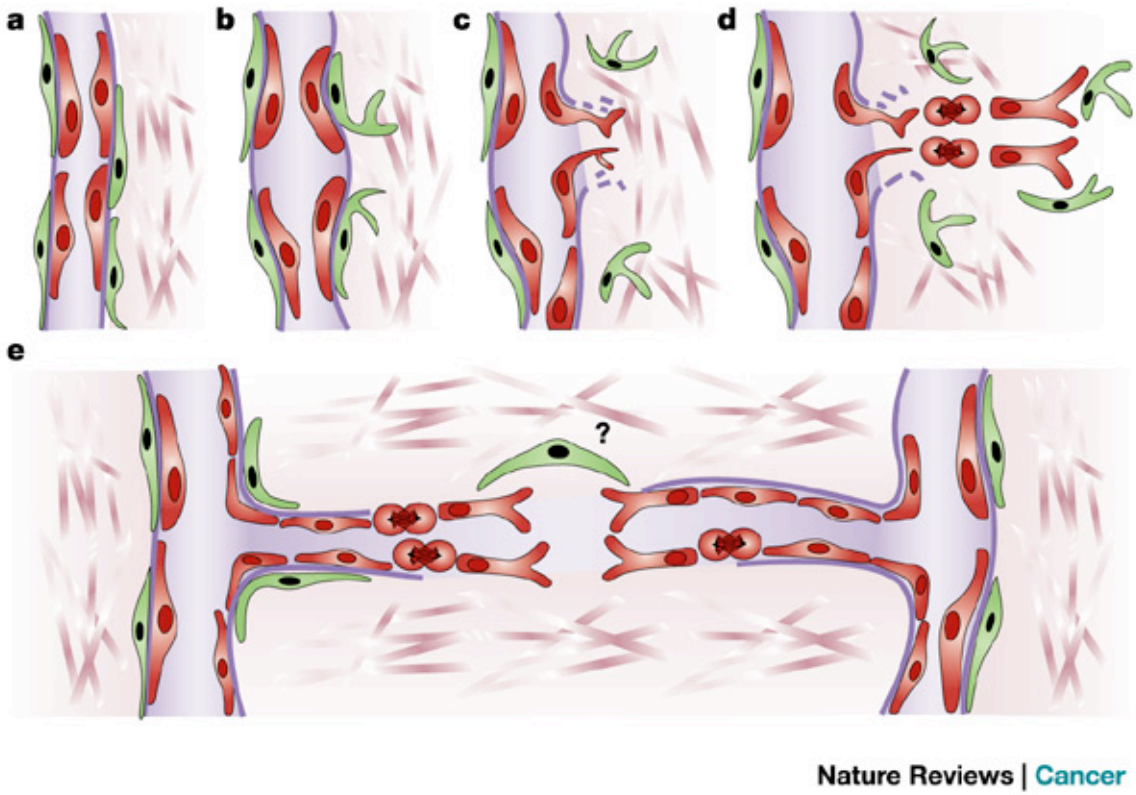


Figure 2. New blood-vessel formation. (a) Blood vessels arise from pre-existing capillaries or post-capillary venules. (b) First, pericytes (green) detach and blood vessels dilate before the basement membrane and extracellular matrix is degraded. (c) This allows endothelial cells (red) to migrate into the perivascular space towards angiogenic stimuli. (d) Endothelial cells proliferate, loosely following each other, and are presumably guided by pericytes. (e) Behind the migration columns, endothelial cells adhere to each other and create a lumen, which is accompanied by basement-membrane formation and pericyte attachment. Finally, blood-vessel sprouts will fuse with other sprouts to build new circulatory systems. Little is known about this fusion mechanism. Figure by Bergers et al. [137]

1.1.3. Angiogenesis in the heart

Some pathophysiological changes in diseased hearts predispose for a deficient oxygen supply, such as an altered architecture of capillaries and arterioles (*microvascular rarefaction*) [13], decreased angiogenesis [9,14], ventricular dilation and a longer diffusion distance between blood vessel and myocardial cells because of fibrosis and matrix apposition [14]. All of these changes can lead to a decreased blood flow. Increased myocardial demands must be met by an equivalent increase in blood flow or vascular supply [9]. This is especially the case where oxygen demand and supply are unbalanced due to either an enlargement of myocardial mass, i.e., heart hypertrophy of any kind, or occlusive coronary artery disease. The resulting myocardial ischemia necessitates an improvement of the vascular supply by

emerging collaterals, which protect the myocardium from ischemic damage [9]. Experiments with artificially induced myocardial infarction and exogenous induction of neovascularization with FGF in healthy canine hearts [15] and chronic ischemic porcine hearts [16] suggest that angiogenesis contributes to the preservation of ischemic tissue and myocardial pump function in evolving myocardial necrosis. Therefore, therapeutic angiogenesis has emerged as a promising new method of treatment for patients with coronary artery disease or ischemic heart disease [11].

1.1.4. Angiogenesis in cancer tissue

Tumor growth is often a multi-step process that starts with the loss of control of cell proliferation in cancerous cells. The cancerous cells begin to divide rapidly and as the tumor mass grows, the cells will find themselves further and further away from the nearest capillary [17]. Finally, the tumor stops growing. The restriction in size is caused by a lack of nutrients and oxygen. In other words in a tumor the angiogenic phenotype can be triggered by the increasing distance of the growing tumor cells to the capillaries or from the inefficiency of the newly formed vessels to sustain such growth [17,18]. Thus tumors can switch to an angiogenic phenotype, meaning increased secretion of angiogenic factors by solid tumors and reduction of negative regulators of angiogenesis [19] (Figure 3). Excessive angiogenesis developing mostly in response to hypoxia will contribute to the pathology [20]. In both normal and pathological angiogenesis, hypoxia is the main force initiating the angiogenic process. Inhibition of angiogenesis can prevent diseases with excessive vessel growth such as cancer. In tumor growth, cancer-causing genetic changes, possibly in conjunction with environmental influences, are able to promote angiogenesis. The pivotal role of angiogenesis in tumor progression and metastasis has encouraged the researchers to test newly developed inhibitors of angiogenesis in a broad variety of animal tumor growth models.

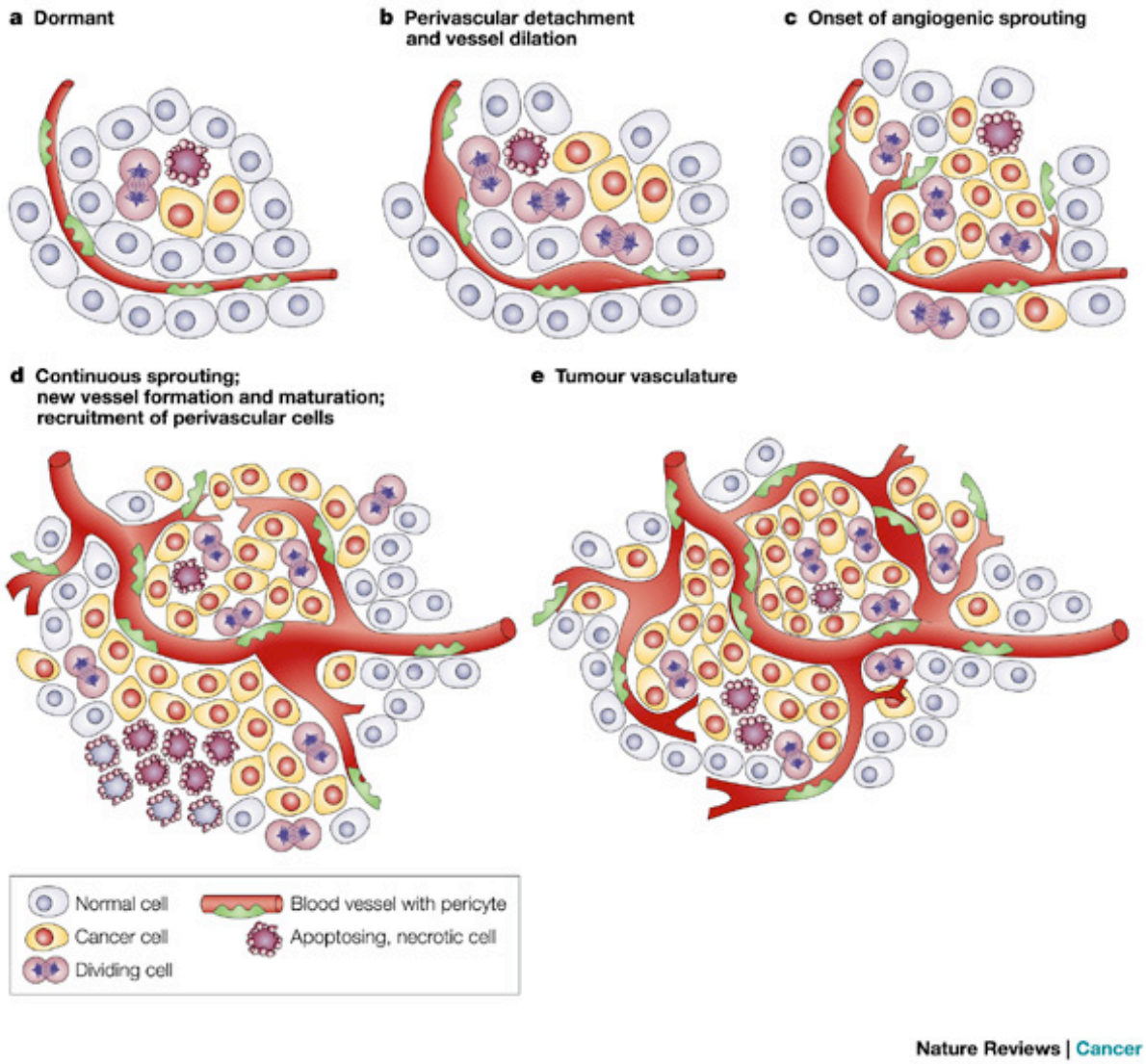


Figure 3. The classical angiogenic switch. The angiogenic switch is a discrete step in tumour development that can occur at different stages in tumour-progression, depending on the nature of the tumour and its microenvironment. Most tumours start growing as avascular nodules (dormant) (a) until they reach a steady-state level of proliferating and apoptosing cells. The initiation of angiogenesis, or the 'angiogenic switch', ensures exponential tumour growth. The switch begins with perivascular detachment and vessel dilation (b), followed by angiogenic sprouting (c), new vessel formation and maturation, and the recruitment of perivascular cells (d). Blood-vessel formation will continue as long as the tumour grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumour to provide it with essential nutrients and oxygen (e). Figure by Bergers et al. [137].

1.2. Hypertension and angiogenesis

1.2.1. The heart during hypertension

Cardiovascular disease usually starts with the classic risk factors such as obesity, diabetes, smoking, dyslipidemia and hypertension (Figure 4) [21]. Arterial hypertension is a very prevalent, important risk factor not only for cardiovascular, but also renal, and cerebral diseases and dementia. Arterial hypertension is also strongly associated with left ventricle hypertrophy (LVH), which is an independent risk factor for cardiovascular morbidity and mortality. In the early stages of hypertension, left ventricle (LV) structure and function will typically be more normal [22-25].

Over time, the pathologic effects of one or more cardiovascular risk factors may cause LV hypertrophy (LVH) to develop or a myocardial infarction to occur. LVH is characterized by rarefaction of microvessels and in consequence with ischemia of the left ventricular myocardium. LV remodeling leads to systolic or diastolic dysfunction, which can further develop symptomatic heart failure.

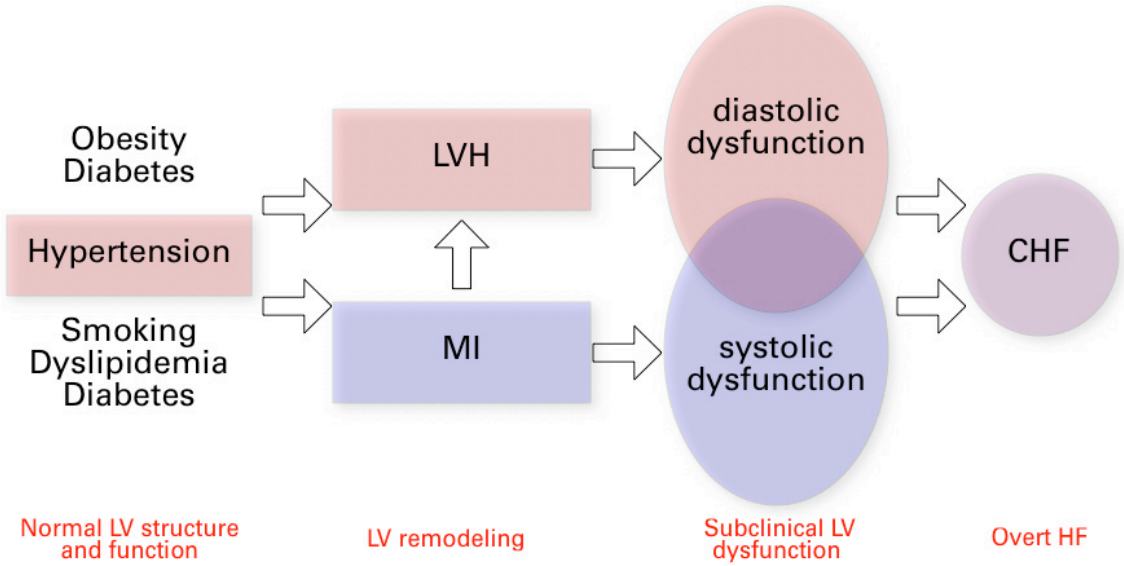


Figure 4. Progression from hypertension to heart failure. CHF = congestive heart failure; CV = cardiovascular; HF = heart failure; LV = left ventricular; LVH = left ventricular hypertrophy; MI = myocardial infarction.

1.2.2. Hypertension and microvascular rarefaction

Arterial Hypertension is associated with altered function and structure of large and small vessels. Abnormal regulation of vasomotor tone, enhanced vasoconstriction, reduced vasodilation, structural alterations of arteries, microvessels and microvascular networks. These changes contribute substantially to hypertension and hypertension-associated target organ [26,27]. For example microvascular rarefaction contributes to increased peripheral vascular resistance and in consequence to the development of chronic arterial hypertension [12]. Prewitt and others [12,28-31] suggested that rarefaction develops after the blood pressure begins to rise. The arterioles first go through a period of functional rarefaction where they are closed to flow but can be opened with vasodilators. Later, the closed vessels are lost completely. This occurs in spontaneous hypertensive rats (SHR) as well as in renal hypertensive models where there is no genetic predisposition for rarefaction. Struijker-Boudier and others [12,31-33] refined these findings. They found that rarefaction can indeed be resulted from a persistently elevated blood pressure and substantiated this in many models of secondary hypertension. However, in primary (genetic) forms of hypertension rarefaction can occur at very early stages before significant elevation of pressure. This was shown in the SHR, but also in human essential hypertension [12,26] (Figure 4).

1.2.3. Hypertension and impaired angiogenesis

In consideration of the studies by Struijker-Boudier and Prewitt [12,29,31,33-35], the term "rarefaction" should be specified as "primary" when it occurs at early stages of hypertension and is related to decreased angiogenic capacity and "secondary", due to pressure increase. Hypothetically, impaired angiogenesis, i.e., inadequate formation of new blood vessels in patients prone to hypertension may evolve because of genetic disposition, deficient placental and embryonic vascular development, and thus impaired postembryonic vascular growth in general and in target organs (for example in hypertrophying myocardial tissue) [27]. This deficiency in the growth of arterioles or capillaries contributes to an increased peripheral vascular resistance and persistence of hypertension [36]. Furthermore, it may be intrinsically associated with development of hypertension-dependent target organ damage [9,10,12,31] (Figure 4).

1.2.4. Reversing impaired-angiogenesis in hypertension

Up to now the main focus in antihypertensive therapy was to induce vasodilation [31]. However, attention has recently also been directed at reducing or even reversing microvascular rarefaction [31]. Several antihypertensive drugs, which were initially designed to promote vasodilation, are now known to improve altered structure of arteries and microvascular networks. Latter effects require time whereas changes of vascular tone occur quickly. To reverse microvascular rarefaction by antihypertensive therapy, microvascular networks, which have been destroyed in response to high blood pressure or which have not formed because of hypertension-associated impaired angiogenesis need to be established or re-established.

1.2.5. Keypoints hypertension and angiogenesis

Theoretically hypertension due to microvascular rarefaction and impaired angiogenesis can be reversed: 1) microvascular rarefaction precedes manifest elevation of blood pressure and hypertension in persons with a family history of hypertension and in animal models of hypertension [26]; 2) nitric oxide (NO) biosynthesis and the Renin-angiotensin-aldosteron-system (RAAS) play pivotal roles in the development of hypertension, and both regulatory pathways affect angiogenesis substantially [37]; 3) induction of arterial hypertension by NO-biosynthesis inhibitors leads to impaired generation of a vascularized connective tissue *in vivo*, i.e., impaired angiogenesis [38]; and 4) antihypertensive treatment can reverse microvascular rarefaction in animal models of hypertension *in vivo*.

1.3. The Renin-angiotensin-aldosteron system (RAAS) and angiogenesis

1.3.1. Activation of the Renin-angiotensin-aldosteron system (RAAS)

The Renin-Angiotensin-Aldosteron System (RAAS) is involved in the pathophysiology of hypertension [39,141]. The RAAS plays an important role in the hormonal mechanisms that regulate blood pressure [39]. Factors that reduce blood volume, renal perfusion pressure or plasma sodium concentration activate this system, whereas increases in these variables suppress the pathway [40]. The activation of this cascade is initiated by renin, which is released from the kidney. Renin cleaves angiotensinogen, produced in the liver, to angiotensin I (Ang I). This latter molecule is further processed to angiotensin II (Ang II) [39,41,42]. Ang II is the main effector molecule of the RAAS. It is an octapeptide with potent vasoconstrictor properties [41]. Ang II promotes salt and water retention and cell growth in vascular and myocardial tissue [43-45] and appears to act as an angiogenic factor [46]. However, the exact mechanisms by which Ang II induces angiogenesis are not fully elucidated yet. Ang II is conversed from its inactive precursor, the decapeptide Ang I, to its active form Ang II by Angiotensin-Converting-Enzyme (ACE). ACE, a carboxypeptidase enzyme released from the lungs, plays a major role in the regulation of the vascular tone by converting the Ang I into the vasoconstrictor Ang II. ACE, also known as kininase, is at the same time the enzyme responsible for degradation of Bradykinin (BK), a potent vasodilator [47]. The effect of the RAAS on blood pressure is also modulated by interaction with other vasoactive systems including the Kininogen-kallikrein- Bradykinin -system.

1.3.2. Angiotensin-converting-enzyme inhibitors

Angiotensin-conversion-enzyme (ACE) inhibitors, which block the conversion of Angiotensin I to Angiotensin II, are used primarily to treat high blood pressure and congestive heart failure [41]. ACE inhibitors have been demonstrated to reduce myocardial injury in cell culture, in isolated hearts [48], and in animal models [49], all of which were subjected to ischemic conditions. A study with the ACE inhibitor quinaprilat in ischemic rat limbs *in vivo* demonstrated that ACE inhibition increases capillary density in ischemic tissue by the induction of new microvessels [50]. Fabre et al. suggested that quinaprilat promotes angiogenesis in a rabbit model of hindlimb ischemia *in vivo* [50]. More clinically-oriented *in vivo* models show that the ACE inhibitor perindopril increases vessel density and capillary number in ischemic hindlimbs of mice [51]. Spirapril, another ACE inhibitor, substantially increases

myocardial capillary microvascular density in spontaneously hypertensive rats [52]. Spirapril also improves left ventricular function by reducing its thickness and its hypertrophied weight [52], which might be due to enhanced angiogenesis. These beneficial actions were indistinguishable from exogenous Bradykinin suggesting that they may not only be due to ACE inhibitors action to decrease Angiotensin II levels, but also to the inhibition of degradation of Bradykinin [47,48,53-55]. Indeed with the availability and use of potent Bradykinin antagonists it was shown that the effect of the ACE inhibitors was abolished, suggesting that inhibition of the degradation of kinins is the overriding protective mechanism [47,56,57].

1.3.3. Kallikrein-kinin-system (KKS)

Kinins belong to a group of 9-11 amino acid peptides, including Bradykinin (BK), kallidin and des-Arg-kinins. These molecules participate in inflammatory processes by virtue of their ability to activate endothelial cells. They also lead to vasodilation, increased vascular permeability, and production of nitric oxide. Kinins also stimulate sensory nerve endings [58,59]. Thus the classical parameters of inflammation (i.e., redness, heat, swelling, and pain) can all result from kinin formation [58]. Kinins are released from plasma precursors, the kininogens, by the action of kallikreins [47,60]. Plasma kallikrein releases Bradykinin from high molecular weight kininogen (HMWK) and tissue kallikrein releases Lys-Bradykinin from low molecular weight kininogen (LMWK). The KKS is activated by most diverse stimuli including ischemia, tissue damage or inflammation [47]. Kinins undergo rapid metabolic degradation by several enzymes including angiotensin-converting-enzyme [59] (Figure 5).

Kinin peptide levels are increased in the heart of rats with myocardial infarction and in tissues of spontaneously hypertensive rats, suggesting a role for kinin peptides in the pathogenesis of these conditions [61]. Kinins induce various signal transduction mechanisms including the activation of phospholipase A2, C and D and the subsequent release of nitric oxide, inositol phosphates and diacylglycerol, leading to the mobilization of several protein kinase C isoforms [59]. Despite the early detection of kinins, many aspects of their physiology and their role in diseases are yet to be defined [61].

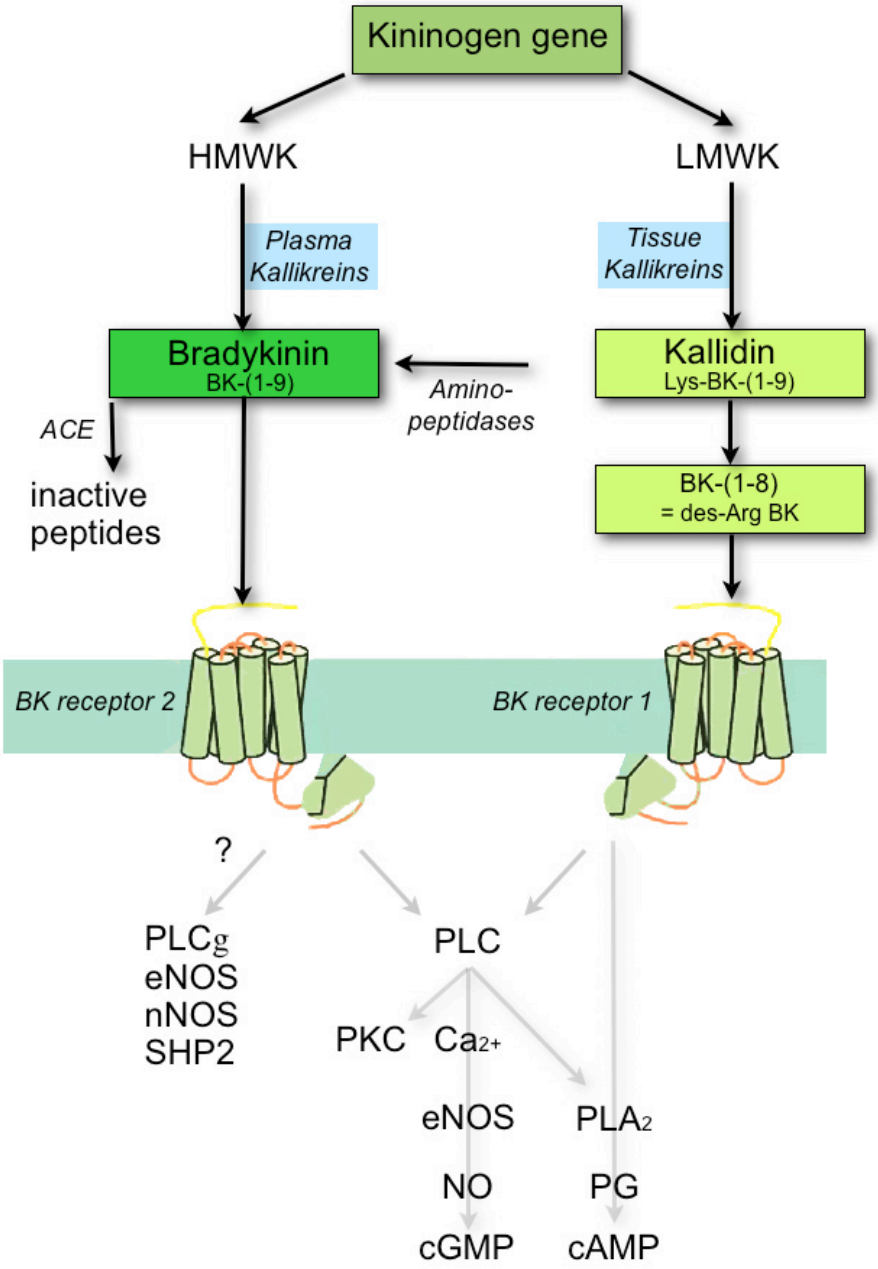


Figure 5. The kinin pathway. Generation of kinin peptides by tissue and plasma kallikrein. In tissue kallikrein generates kallidin whereas plasma kallikrein generates Bradykinin (BK) from LMWK and HMW, low and high molecular weight kininogens. Whereas Bradykinin [BK-(1-9)] and kallidin [Lys-BK-(1-9)] are more potent agonists for the BKR2, BK-1-8 and kallidin-(1-8) are more potent agonists of the BKR1. Signaling via Bradykinin receptors involves: Phospholipase Cg (PLCg), nitric oxide (NO), endothelial and neuronal NO synthase (eNOS, nNOS), src homology domain 2 (SHP2), phospholipase A2 (PLA2), prostaglandin (PG), protein kinase C (PKC), cyclic guanidin and adenosin monophosphate (cGMP, cAMP) and others. Figure created based on Kaplan et al.[138, 139, 140]

1.3.4. Bradykinin (BK) and BK receptors

Bradykinin is a potent short-lived vasodilator. Bradykinin is formed by tissue Kallikrein from its endogenous protein substrate kininogen [62]. Bradykinin participates in inflammatory and vascular regulation, including the regulation of blood pressure, angioedema, tissue permeability, and smooth muscle contraction [63,64]. The actions of Bradykinin are mediated through two receptor subtypes; BKR1 and BKR2. Recently, a third Bradykinin-activated receptor has been described, GPR100 [65]. These receptors belong to the family of G-protein-coupled-receptors (GPCRs) [63]. Bradykinin often exerts its biological effects through the activation of the BKR2, which is generally constitutively expressed and predominates [63]. In contrast BK1 receptors are induced by tissue injury [61], myocardial ischemia [66] and inflammation [63]. However, there is some evidence suggesting that the BKR1 exerts effects also when expressed minimally (constitutively) [63,67]. Besides the classical pathways, mentioned above, the BKR2 is also linked to the activation of protein tyrosine kinase as well as MAP kinase. Conversely, the BK1R is primarily linked to the activation of phospholipase C [59].

1.3.5. Bradykinin in reparative angiogenesis

ACE inhibition leads to accumulation of Bradykinin. The beneficial effect of ACE inhibition on the microvasculature is probably due to angiogenesis via Bradykinin and other molecules such as FGF, VEGF, eNOS and PKC [68-71]. Moreover, the angiogenic effect of ACE inhibition appears to be mainly mediated via Bradykinin and the BK2 receptor. Thus, in an ischemia-reperfusion rat heart model, ACE inhibition partially reduced myocardial infarction and apoptosis via the BKR2 receptor [72]. In a model of surgically induced hindlimb ischemia in mice, ACE-inhibition leads to angiogenesis via BKR2 signaling and upregulation of endothelial nitric oxide synthase (eNOS) [51]. BKR2 knockout mice display reduced capillary density [73]. Moreover, Bradykinin promoted angiogenesis via BKR2 by increasing vascular and endothelial permeability and by up-regulation of VEGF via BKR2 [74]. Confusingly, other reports suggest that the BK1 receptor is required for the angiogenic response to Bradykinin. For example, the BK1 receptor is upregulated in ischemic skeletal muscle of mice [75] or ischemic myocardium of rats [76]. In line with this, abrogation of BK1 receptor signaling inhibits an angiogenic response in a murine model of hindlimb ischemia [55]. Conversely, delivery of BK1 receptor agonist enhances collateral vascular growth in ischemic skeletal muscle of mice [55]. *In vitro* BK1 receptor activation stimulates endothelial cell

proliferation and survival [55]. Bradykinin acting via the BK1 receptor up-regulates the angiogenic factor FGF-2 via the iNOS pathway [68,77]. Taken together, Bradykinin seems to be a powerful angiogenic stimulus *in vivo* and *in vitro*. Reports describing mechanisms of how Bradykinin induces angiogenesis are diverse. In particular it is not clear which Bradykinin receptor subtypes elicit a pro- or antiangiogenic response[77]. Furthermore, different effector molecules or mechanisms responsible for Bradykinin-induced angiogenesis have been described such as upregulation of VEGF, bFGF or transactivation of VEGFR2 [68,77,78] .

1.3.6. Bradykinin in tumor angiogenesis

The nanopeptide Bradykinin is an important growth factor for many cancers as certain Bradykinin antagonists show remarkable anti-cancer activities [79]. Most solid tumors are known to exhibit highly enhanced vascular permeability, which may supply nutrients as well as oxygen. This permeability may be due to the presence of Bradykinin [18]. In *in vitro* studies tumor cells can generate Bradykinin, which is also present in blood plasma and pleural fluids of cancer patients [18,80,81]. *In vivo* Bradykinin promotes angiogenesis in mice bearing sarcoma 180 cells by increasing vascular permeability and by promoting up-regulation of VEGF [74]. In these mice daily administration of BK2R antagonist suppresses the increase in angiogenesis and tumor weight.

1.4. The hypoxic heart

1.4.1. Hypoxia and angiogenesis of the heart

Hypoxia is a potent regulator of a variety of biological processes, including angiogenesis and vascular contractility. The heart is hypoxic, i.e., the myocardium is ischemic when a major coronary artery occludes or when a deficient growth of the microvasculature can not keep pace with the rate of hypertrophying myocardium [82]. At this stage the vascular supply is overwhelmed by increasing metabolic demands [83], and any accelerated heart rhythm will enhance oxygen consumption. This leads necessarily to a state of hypoxia in the microenvironment [83].

Angiogenesis serves to increase blood supply to insufficiently oxygenated organs [11,14]. Responses to hypoxia can be acute, occurring over a period of seconds to minutes, or chronic, with a time course of hours to days [84]. Hypoxia is an important stimulus of SMC and EC proliferation and is found in atherosclerotic lesions and rapidly growing tumors [84]. Thus hypoxia can upregulate numerous genes that trigger neovascularization, proliferation and remodeling within the vascular wall [84,85]. Hypoxia activates hypoxia-inducible factor 1 (HIF-1) which is a transcriptional factor and is expressed in response to a decrease in the partial pressure of cellular oxygen. HIF-1 activates genes involved in angiogenesis. Under conditions of hypoxia, HIF-1 is stabilized by inhibition of prolyl hydroxylase, thus preventing HIF's proteolytic degradation. Recently, signaling via mTOR was also shown to stabilize HIF-1 [144]. Hypoxia activates vascular endothelial growth factor (VEGF) [145, 146] or nitric oxide synthases [144] via HIF-1. Furthermore, an experimental study on benefits of ACE-inhibitors have shown that hypoxia significantly increases Bradykinin levels in rat cardiac myocytes [86]. In line with this, altered production and release of potent vasoactive substances by the endothelium under hypoxic conditions, such as Bradykinin, is suggested to be involved in the development of pulmonary vascular remodeling [141].

1.5. Nitric oxide (NO)

1.5.1. NO and NO synthases

NO acts as a mediator in the vascular, nervous, and immune systems by regulating vascular permeability, vasodilation, tumor blood flow, platelet adhesion and aggregation, and other functions [87,88]. Many of the biological effects of NO are mediated via the activation of guanylyl cyclase which increases cGMP formation from GTP [89,90]. The synthesis of NO is catalyzed by a group of enzymes called Nitric Oxide Synthases (NOS) [91,92]. Three NOS isoforms have been identified which are named according to the cell type or conditions in which they were first detected: neuronal NOS (nNOS or NOS1), inducible or inflammatory NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) [91,92].

nNOS is highly expressed in the central and peripheral nervous system and in skeletal muscles [92-95]. In the central nervous system, nNOS-derived NO may be an important mediator of behavioral inhibition. In peripheral nerves NO derived from nNOS is important in the relaxation of vascular and non-vascular endothelial cells [92,95].

eNOS was reported to be expressed in various cells like cardiac myocytes, glial cells, and brain cells (hippocampus) [92,94]. eNOS expression is usually reported to be constitutive though modest degrees of regulation occur in response to factors such as shear stress, exercise training, chronic hypoxia and heart failure [92,94]. NO derived from eNOS in cardiovascular system maintains the vasculature in a relaxed state, inhibits adhesion of platelets and white cells, and suppresses replication of smooth muscle cells [92]. eNOS knock out mice are hypertensive and more prone to atherogenesis. eNOS also promotes angiogenesis and regulates VEGF expression [92,96].

iNOS is expressed in a large number of cells. However, it is expressed only after induction by immunologic or inflammatory stimuli, [92,94,97,98]. Once expressed, iNOS generates large amounts of NO often to a detrimental level [91,92,97]. Induction of iNOS is found in models of septic shock, inflammatory and non-inflammatory pain, arthritis, asthma, in the brain after ischemia or trauma, and in various models of neurodegeneration or cerebral inflammation [92,97]. iNOS is important in skin wound healing and healing of intestinal mucosa, is involved in angiogenesis (see later) , and is a key mediator in ischemic preconditioning [92,97].

1.5.2. NO and angiogenesis

Reduced NO bioavailability plays a central role in the development of arterial hypertension, and NO is required for angiogenesis *in vivo* and growth-factor-mediated endothelial tube formation *in vitro*. Vasodilation precedes sprout-formation at initiation of angiogenesis [7,99]. This vasodilation may be NO-mediated, and NO-mediated vasodilation may be a prerequisite of elongation, migration, and proliferation of endothelial cells. All of these cellular actions are required for angiogenesis [99]. Both inducible NOS (iNOS) and endothelial NOS (eNOS) have directly been implicated in wound healing, where angiogenesis is an integral part of the process [100-102]. Furthermore, NOS inhibitors delay wound healing [147].

1.6. RATIONALE & AIMS

Myocardial ischemia results from left ventricular hypertrophy (LVH) in hypertension or coronary heart disease. It is one of the most important reasons for morbidity and mortality in the Western world. Besides other features, LVH is characterised by rarefaction of microvessel density. Great interest has been focused on improving ischemia of the left ventricular myocardium by use of angiogenic growth factors such as vascular endothelial growth factor and fibroblast growth factor.

Inhibition of angiotensin converting enzyme (ACE) is an efficient treatment of persistent high blood pressure, vasoconstriction and LVH. A number of studies have recently uncovered an unexpected effect of ACE inhibitors; they increase microvessel density in peripheral tissues and thus help to reoxygenate the affected ischemic tissue.

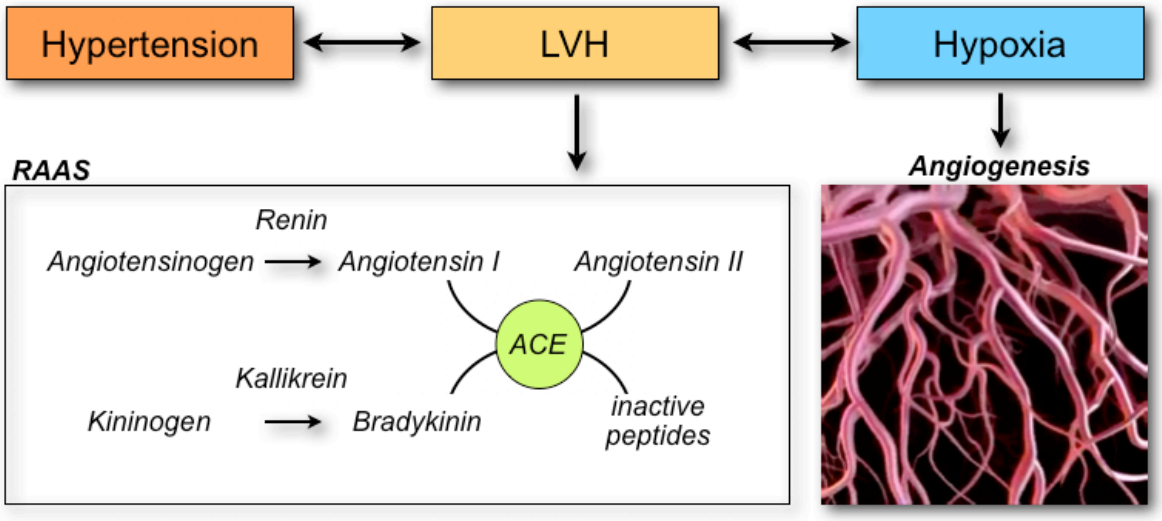


Figure 6. Left ventricular hypertrophy (LVH) in arterial hypertension is associated with microvascular rarefaction. Furthermore, left ventricular hypertrophy is also associated with tissue hypoxia and activation of Renin-angiotensin-aldosteron-system (RAAS). As a result of activation of the RAAS we find increased Bradykinin degradation. Increasing Bradykinin concentrations with angiotensin-converting-enzyme-inhibitors may contribute to regenerative angiogenesis.

Recent reports suggest that the cardioprotective effect ascribed to ACE inhibitors at a cardiovascular and tissue level might be due to the proangiogenic effect of the vasodilator Bradykinin (BK), which is found at increased concentrations following inhibition of its degradation by ACE inhibitors (Figure 6). However, reports describing

mechanisms of how Bradykinin induces angiogenesis are divergent. In particular it is not clear, which Bradykinin receptor subtype elicits a pro- or antiangiogenic response. Furthermore, different effector molecules or mechanisms responsible for Bradykinin-induced angiogenesis have been described. It is also unclear, which signalling mechanisms mediate angiogenesis in the heart during ischemia and hypoxia.

The aim of this study was to assess the potential of ACE inhibition, in particular the potential of Bradykinin as a proangiogenic factor in therapeutic angiogenesis of the heart (see also Figure 6). We also aimed better understand how Bradykinin induces angiogenesis at the molecular and cellular level.

Specifically we wanted to determine:

(1) The role and presence of Bradykinin receptor subtypes (BKR1, BKR2) in heart angiogenesis *in vitro*

(2) The down stream, key signaling relay enzymes

(3) The effector molecules of Bradykinin-induced angiogenesis

(4) The role of Bradykinin in tumor angiogenesis *in vitro* in comparison to heart angiogenesis *in vitro*.

To answer these questions we used an *in vitro* assay of angiogenesis that we developed to study microvessel formation in rodent hearts. The assay allows investigating tissues from wildtype and genetically modified mice, the use of specific pharmacological antagonists and agonists, as well as the assessment of the role of diminished oxygen saturation.

2. MATERIALS & METHODS

2.1. *In vitro* assay of angiogenesis

2.1.1. Assay description

This assay was developed in our laboratory with the aim of developing a new *in vitro* angiogenesis assay of the heart based on a classical three-dimensional assay of angiogenesis *in vitro* using rat aortic explants [103]. In this assay, pieces of rat aorta are cultivated in fibrin or collagen gels. After a week, endothelial cells start to grow out from the piece of aorta and form sprouts. This outgrowth can be analyzed morphometrically and corresponds to angiogenesis *in vitro* [84,103]. For this *in vitro* angiogenesis model we used small pieces of rat or mouse heart instead of aorta (Figure 7). The standard conditions were established in our laboratory for *in vitro* heart angiogenesis in mouse and rat hearts (Kiefer et al. 2004, Exp Cell Res, *in press*).

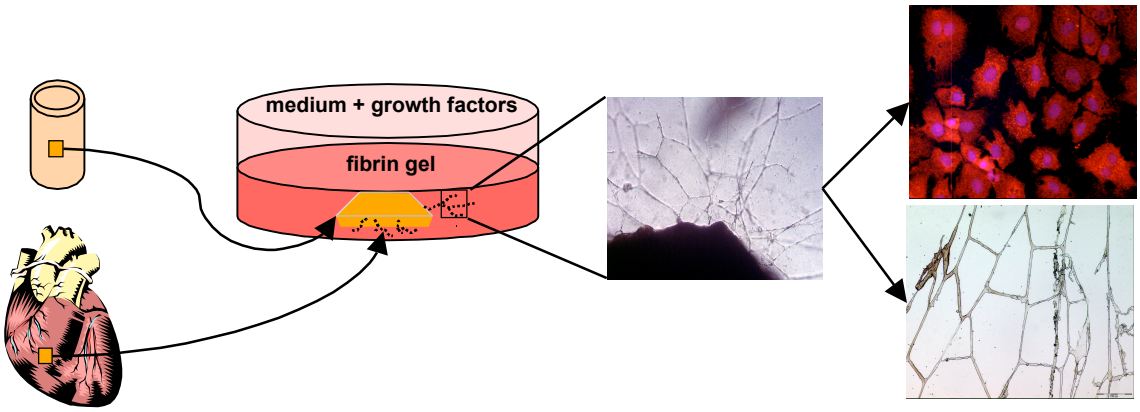


Figure 7: Model of angiogenesis *in vitro*: This assay can be used to assess angiogenesis in a variety of tissues, including aortae, hearts and tumor tissue. Endothelial sprouts can be further analyzed by immunohistological staining. Outgrowing rat but not mouse endothelial cells can be subcultured for *in vitro* assays.

Briefly, a fibrin gel solution was prepared by mixing 3 mg of fibrinogen (Sigma-Aldrich, Buchs, Switzerland) per ml serum-free DMEM (Oxoid, Basel, Switzerland)

with 0.1 U/ml of thrombin (Sigma-Aldrich, Buchs, Switzerland) on ice. 100 µl aliquots of this fibrin gel solution were immediately pipeted into each well of 48-well plates and allowed to polymerize for one hour at 37°C. Gels were then overlaid with 500 µl serum-free DMEM for at least 30 minutes. Medium overlaying the gel was removed and 1mm^3 cubes from the myocardium of the left ventricle were placed onto the gels in each well and overlaid with 100 µl of fibrin gel solution. After one hour of polymerization, gels were overlaid with 500 µl standard DMEM containing 5% fetal calf serum (FCS, Oxoid, Basel, Switzerland). Heart explants were then exposed to agonists and/or antagonists incubated under normoxic (21% O_2) and hypoxic (3% O_2) conditions for 10 days, with replenishment of agonists and/or antagonists every second day. Fibrin gels were protected from degradation by adding 300 µg/ml e-amino-caproic acid (Sigma-Aldrich, Buchs, Switzerland) every second day. After 10 days endothelial sprouts were photographed digitally (ColorView II, Soft Imaging System, Gloor Instruments, Uster, Switzerland) on an inverted light microscope (Olympus IX50, Olympus, Schwerzenbach, Switzerland).

Evaluation of the culture medium:

To find optimal culture conditions we investigated standard cell culture medium (Dulbecco's minimal essential medium, DMEM) supplemented with different concentrations of fetal calf serum (FCS; 0, 1, 2,5, 5 and 10% FCS) after 10 days of incubation. DMEM supplemented with 5% FCS proved to be the optimal combination for our *in vitro* heart angiogenesis assay. Unstimulated control cultures, i.e., containing 5% FCS, displayed weak sprouting that was additionally amplifiable by addition of growth factors (Kiefer et al. 2004, Exp Cell Res, *in press*).

Time course of angiogenesis of the heart:

The initial incubation time of 10 days for the *in vitro* angiogenesis assay of the heart was set arbitrarily. Under conditions of hypoxia first sprouts were observed after 3 days of incubation. After 10 days sprouting was maximal. Under normoxia sprouting was weak and first sprouts started to emerge after 5 days and did not develop further.

2.1.2. Quantification of sprout formation

In order to evaluate sprout formation we used two different methods: The angiogenic index and the AnalySIS software. However, we primarily used the

angiogenic index since the resulted data with this index were reproducible and the method showed a greater simplicity than the AnalySIS software. Nevertheless, we consistently used the image analysis to corroborate the angiogenic index. Both methods showed very similar results.

2.1.2.1. The angiogenic index

For the angiogenic index, pieces of heart were digitally photographed at 4fold magnification. We evaluated sprout formation with the use of an empiric scale ranging from 0 to 8. 0 represents no growth and 8 represents a fully overgrown visual field. For scale used see figure 8 on page 30.

2.1.2.2. The AnalySIS software

We quantified the outgrowing sprouts with the AnalySIS software by measuring the surface of area covered by outgrowing cells in relation to the surface of the piece of heart (Figure 9). We did not include any form factor. Thus, we did not differentiate whether cells were organised or not. Our primary goal was to evaluate a variable correlating with the number of cells growing from the piece of heart into the fibrin gel. The AnalySIS software yielded very similar results to those obtained with the angiogenic index. A value of 5 in the angiogenic index corresponds to a value of around 50% in the AnalySIS software. As mentioned above we used the AnalySIS software to control our empirically obtained values with the angiogenic index at regular interval (Figure 9, page 31).

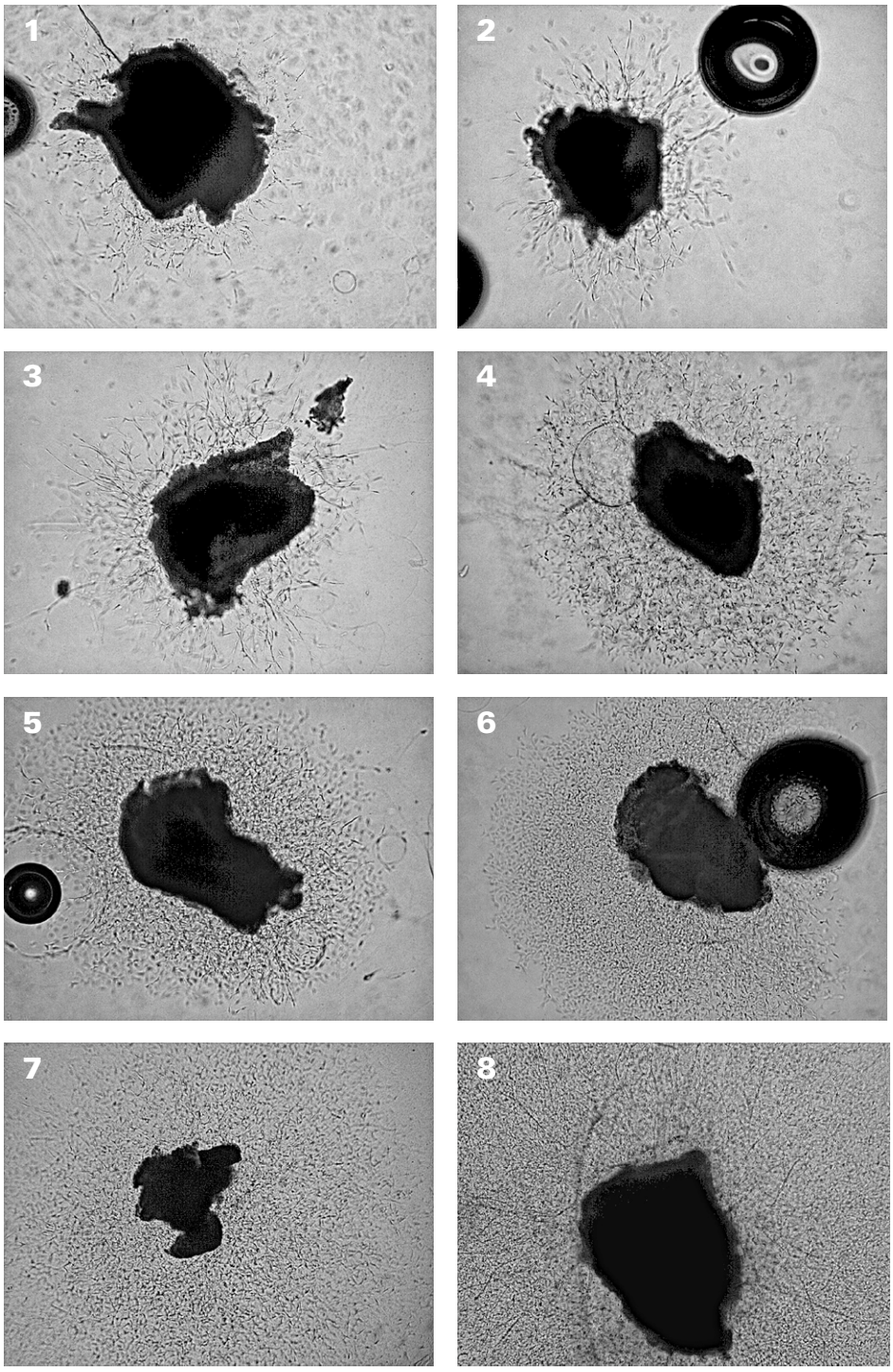
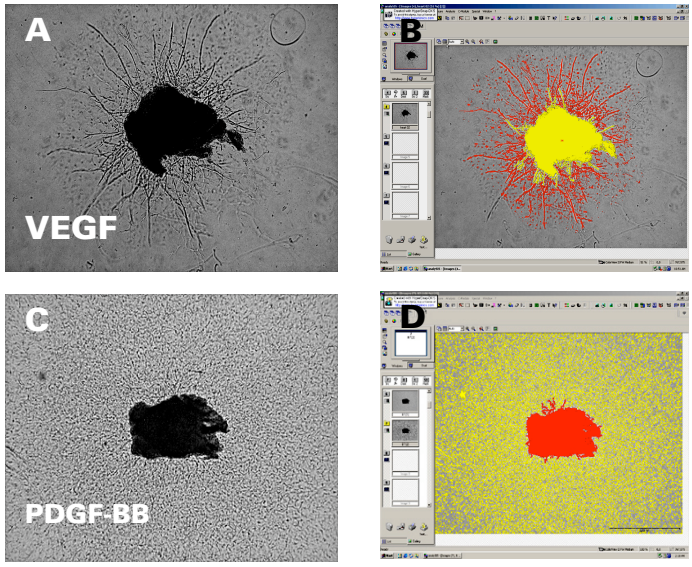


Figure 8. Sprout formation with angiogenic index ranking from 1 to 8. This scale has been used as the standard to estimate angiogenic indices.



Angiogenic Index vs. Analysis									
Angiogenic Index	0	1	2	3	4	5	6	7	8
AnalySIS (%)	0-5	5-15	15-25	25-35	35-45	45-55	55-65	65-75	>75

Figure 9. Use of the AnalySIS software. After 10 days of incubation, pieces of heart were photographed digitally at 4x magnification. The total surface of sprouts and the piece of heart was measured. The fractions of heart and sprouts were set in relation to the total surface. Example (A/B), stimulation with 5 ng/ml VEGF: surface of sprouts is: 814'00 μm^2 and surface of the heart is 152'600 μm^2; total surface is 234'000 μm^2; thus sprouts are 35% of the total surface corresponding to an angiogenic index of 4. For PDGF-BB (D) we would have given a value of 8 in the angiogenic index whereas the AnalySIS software resulted in a surface of 83%. The table shows the relation between angiogenic index and the values obtained with the AnalySIS software.

2.1.3. *In vitro* assay of angiogenesis: Applicability and relevance

This *in vitro* model may serve as a reproducible and reliable tool for analyzing induction as well as repression of angiogenesis in the heart *in vitro*. Our group first tried to reproduce known *in vitro* and *in vivo* phenomena of angiogenesis using this assay.

Growth factors such as PDGF-BB, bFGF or VEGF, that have been proved to induce angiogenesis *in vitro* and *in vivo* [9,108,109] were examined in the *in vitro* system for their ability to form capillary-like sprouts from the embedded heart tissue. Indeed these growth factors induced angiogenesis *in vitro* from pieces of the heart.

A further phenomenon influencing angiogenesis is aging. Age dependency of angiogenesis has been shown in different models of angiogenesis *in vivo* [110,111] and *in vitro* [112,113]. In line with these studies we could show another known phenomenon with our assay: In experiments with 20 week old mice, angiogenesis was even more restricted than in the 12 week old adult mice (Kiefer et al, 2004, Exp Cell Res, *in press*). Given the above mentioned experiments, in which we reproduced known *in vivo* phenomena, we propose that our model is suitable to investigate regulation of angiogenesis of the heart with an easy *in vitro* method.

Most of these experiments were performed in mice and some in rat hearts. Experiments performed in rodents may not always be representative for humans. To clarify this we performed preliminary experiments with small pieces of explanted human left ventricle obtained from a heart transplant recipient and observed a similar angiogenic response *in vitro*. However, these results need to be interpreted cautiously since we had only enough human heart to perform two single experiments. Rodent tissue is much more readily available than human tissue. Apart from the availability, the other great advantage of using mice is that transgenic animals can be used to answer more specific questions. We are aware of the fact that our assay for angiogenic response may not completely reflect myocardial angiogenesis *in vivo* but on the other hand organ culture models may better simulate *in vivo* situations than other assay. They include surrounding non-endothelial cells in their microvascular environment, which pure endothelial angiogenesis *in vitro* assays don't. In addition, endothelial cells have not been pre-selected by passaging and are not in a proliferative state at the

time of experiment and may thus better represent a real-life situation [114]. Hence, sprout formation in our assay could be mediated by direct stimulation of myocardial endothelial cells and possibly also by stimulation of surrounding myocardial tissue which then acts on endothelial cells to promote angiogenesis. Endothelial sprouting in response to angiogenic molecules therefore reflects the integrated interactions of different cell types and the entire myocardial tissue and not the primary response of endothelial cells alone. All our results have been very similar to *in vivo* situations (age, growth factors), and we conclude that results obtained with our assay are likely to represent other situations *in vivo*.

The other advantage of this assay is that it can be used for screening of a broad range of different inducers and inhibitors of angiogenesis. Currently most experiments are performed *in vivo* since no appropriate *in vitro* model is available. *In vivo* experiments require large numbers of animals, are difficult to perform and are often associated with animal pain. Many animals die due to myocardial infarction and cardiac rhythm disturbances during sometimes cumbersome surgical procedures. An appropriate *in vitro* model of angiogenesis of the heart would resolve some of the problems encountered *in vivo* and potentially reduce the number of *in vivo* animal experiments.

Sources of variability. Each single experiment for each single condition needs to be performed in octuplicates in this assay. This relatively high number of identical samples per experimental condition is due to the variability of the assay. We found that 1 to 2 out of 8 pieces of heart do not show any sprout formation independent of the stimulus applied. Sporadically some assays did not work at all. This assay needs a lot of practice and accuracy. The gel layer on top of the heart piece may be too thick or the consistency of the gel too close-meshed – due to a high fibrin concentration. Stimuli and survival factors (FCS) may not diffuse unhamperedly through the gel layer to reach the piece of heart. As a result the heart piece may not survive. Indeed one to two out of ten pieces of heart become necrotic (data not shown, Kiefer et al., 2004, Exp Cell Res, *in press*). On the other hand weak gel layers will induce the pieces of heart to float. A lack of capillary-like sprout formation may also be due to inadequate preparing and cutting of tissue with irreparable damage to the tissue. Also, the time period between embedding of the tissue on the first layer of fibrin-gel and applying the second layer on top of it is crucial. Tissue must be completely encapsulated

in gel within 20-30 minutes. Otherwise samples do not respond, most probably due to necrosis.
Genetic variability may also play a significant role in an individual animals capacity to respond to exogenous angiogenic agents. Therefore, we used hearts from only one specific mice strain (C57BL/6). When examining the effects of transgenes on angiogenesis *in vitro* we used corresponding wild-type mice strains as control.

However about 7 or 6 of the octuplicates show an angiogenic response and only 1-2 experiments does not work out of 30. These variations result in the relatively high standard deviations. This assay is therefore not suited to investigate small differences in the degree of sprout formation between different conditions. In summary, our assay has the potential to be used as a screening tool.

2.2. Agonists and antagonists

Stimulators used were 1 pM-1μM BK acetate (Bradykinin Sigma-Aldrich Chemie GmbH, Germany), 5 ng/ml rat recombinant Vascular Endothelial Growth Factor 164 (VEGF) (hrVEGF164, R&D systems, Minneapolis, MN). List of inhibitors: Inhibitors were added 20 minutes before addition of agonists (BK, VEGF) and were present throughout the 10 days of incubation period. The following pharmacological Bradykinin receptor inhibitors were used: 100 nM BK2 receptor antagonist HOE140 (D-Arg-[Hyp3, Thi5, D-tic, Oic8]-BK); 10nM BK1 receptor antagonist LYS-{des-ARG^{9},LEU^{8}}-BK; 10nM BK1 receptor agonist LYS-{DES-ARG^{9}}-BK. Pharmacological NOS (nitric oxide synthase) inhibitors used were:

100μM L-NAME (Nω-nitro-L-arginine methyl ester), 10μM L-NIL (N 6 –{1-iminoethyl}-lysine) and 1μM L-NIO (L-N 5 –{1-iminoethyl} ornithine). All inhibitors were from Fluka Chemie GmbH, Buchs, Switzerland.

2.3. Immunostaining

For characterization of outgrowing cells and sprouts, specific markers were directly applied to heart cultures. Heart cultures were fixed overnight with 4 % paraformaldehyde (Merck AG, Dietikon, Switzerland), washed with PBS, permeabilized with 0.2% Triton (Fluka Chemie AG, Buchs, Switzerland), washed with PBS once more and incubated for 3 hours with cell specific markers: Alexa

Fluor 448 conjugated GSL I – IB_4 (20 μg/ml; Molecular Probes, USA) for endothelial cells, Cy3-conjugated antibody anti- α smooth muscle actin (1:100; SMA; Fluka Chemie GmbH, Buchs, Switzerland) for smooth muscle cells and pericytes. After incubation with cell markers the probes were extensively washed with PBS. Endothelial sprouts were photographed digitally (ColorView II, Soft Imaging System, Gloor Instruments, Uster, Switzerland) on an inverted light microscope (Olympus IX50, Olympus, Schwerzenbach, Switzerland) (Figure 10).

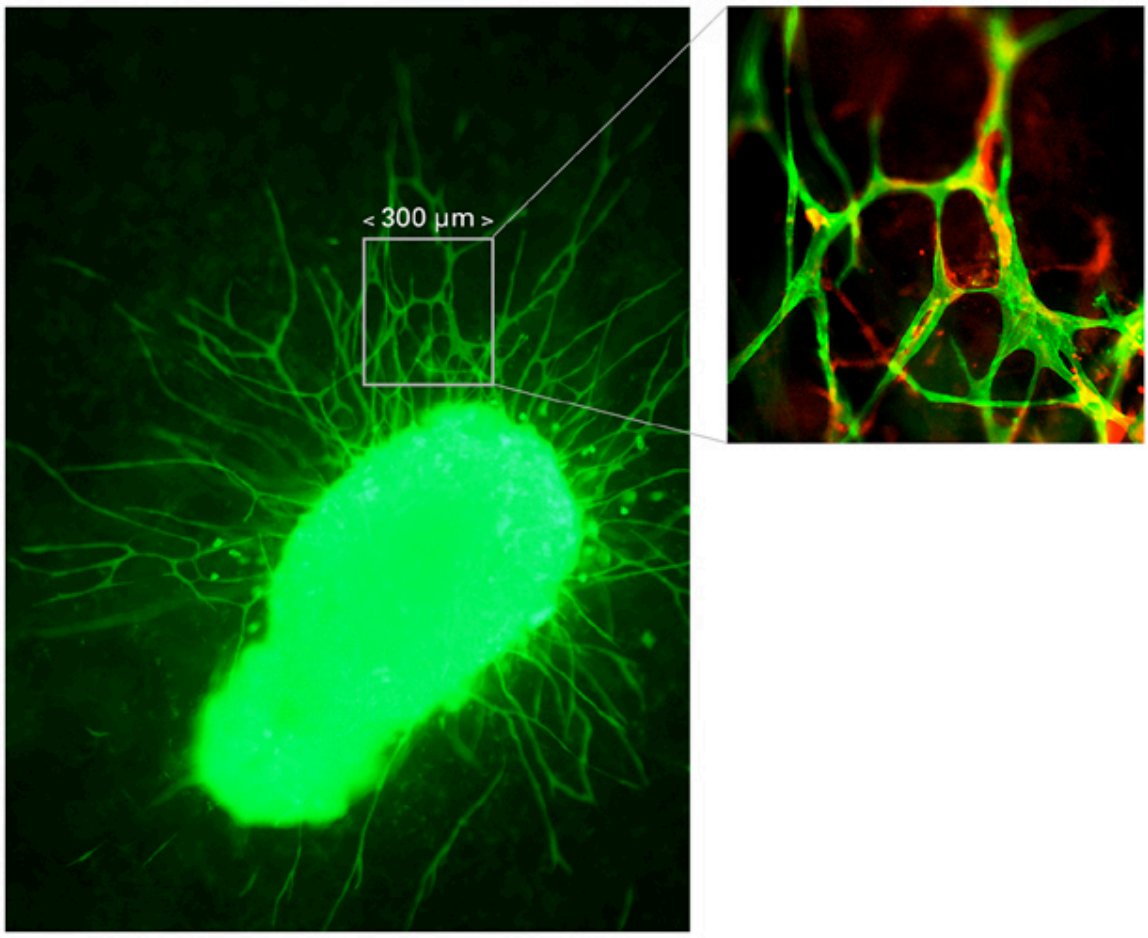


Figure 10: A piece (ca. 1 mm3) of the left ventricular myocardium of a mouse heart is embedded in a fibrin-gel, overlaid with growth medium and angiogenic stimulant (basic Fibroblast Growth Factor, 10ng/ml). After 10 days of incubation, double in-gel-staining with FITC-coupled lectin G. simplicifolia (green fluorescent) and Cy3-coupled antibody against a-smooth muscle actin (red fluorescence) reveal endothelial sprouts with single attached smooth muscle- or pericyte-like cells. Pericyte attachment forming endothelial tubes have been observed *in vivo* and contributes to vessel remodeling, maturation and stabilization (Kiefer et al., Exp Cell Res, 2004, *in press*) [104].

2.4. Animals

Experiments were performed with hearts of C57BL6 male mice. BK1R(-/-) - BL6 mice were from Dr. Jaenette Wood, Novartis Corp., Basel, Switzerland. iNOS(-/-) mouse hearts were from Dr. Christoph von Garnier, Department of Research, and University Hospital Basel, Switzerland [142]. All hearts were obtained post mortem within half an hour after death. Age of iNOS knockout- and OF1-mice ranged from 8 to 12 weeks. All experiments conformed to the rules of the Swiss Federal Act on Animal Protection (1998), and were approved by the Veterinary Department of Basel (Switzerland).

2.5. Tumor model in BK1 (-/-) mice (Procedures described in this chapter were performed by Dr. Amanda Littlewood-Evans & Dr. Jeanette Wood, Angiogenesis Platform Novartis Basel)

Melanomas were injected into the ear of the mice. The B16/BL6 melanoma cell line was obtained from Dr. Isiah J. Fidler, Texas Medical Center, Houston, USA. The cells were cultured at 37°c and 5% CO2 in MEM (MEM EBS, AMIMED, Allschwill) with stable glutamine supplemented with 5% fetal calf serum, 1% sodium pyruvate, 1% non-essential amino acids and 2% vitamins until confluency. Subsequently, they were detached with 0.25% EDTA (2 min at 20°c), and then processed. Viability was assessed by trypan blue exclusion, and only suspensions with >90% viability were used. The tumor cells were re-suspended in Hanks buffer containing 10% FCS, counted and a suspension of 5 x 10^4 cells/µl prepared for intradermal (i.d.) injection in a volume of 1 µl into the dorsal pinna of both ears of BK1 (-/-) mice.

To inject the tumor cells, the mice were anesthetized (3% Isoflurane, Forene ®, Abbott AG, Cham, Switzerland) and then placed onto an operating field maintained at a temperature of 39°c. Their ears were extended over a steel cone fitted with a double-sided sticker to expose the dorsal surface. With the aid of microscope, a 30G hypodermic needle was then inserted in the skin between the first and second neurovascular bundle of the ear and tunneled for 4-5mm. 1 µl of tumor cell suspension (5 x 10^4) cells was injected using a microliter syringe (250 µl, Hamilton, Bonaduz, Switzerland) forming a 2 x 2 mm sub-dermal blister. Primary tumor size was measured in each animal over 3 weeks (days 7, 14 and 21 post tumor cell inoculation) after anaesthetizing the mice (3% Isoflurane) and

viewing the tumor under a light microscope. Treatment was started at day 7, when the tumor was already established. After 2 weeks of treatment (day 21 after cell injection), the animals were killed and the cervical lymph nodes weighed.

2.6. Reverse transcription polymerize chain reaction (RT-PCR)

Heart tissues from mice were placed in standard DMEM containing 5% fetal calf serum. Bradykinin was added at a final concentration of 10nM. After 2, 4, 12 and 24 hours heart tissues were collected and total RNA was isolated with TRIzol Reagent (1ml/1g tissue)(Invitrogen AG, Basel, Switzerland) by homogenizing with tissue homogenizer. Extracted RNA was quantified spectrophotometrically. 1 μg of total RNA from mouse heart tissue was reverse transcribed with M-MLV reverse transcriptase system (Catalys-Promega, Wallisellen, Switzerland).

The cDNA (2μl) was amplified in polymerase chain reactions (PCR). The primer sequences were for mouse BK1 receptor sense: 5′-TGA ACA TCT CTG CCT GCA TC-3′, antisense: 5′-CGG CCT GCA AAG ACA TAA AT-3′; mouse BK2 receptor sense: 5′-CAA CGT CAC CAC ACA AGT CC-3′, antisense 5′-CAC CTCTCC AAA CAC CCA GT-3′; mouse 18S ribosomal RNA sense: 5′-CCT GGA TAC CGC AGC TAG GA-3′, antisense 5′-GCG GCG CAA TAC GAA TGC CCC-3′. Amplification was carried out after an initial denaturation at 94°C for 5 min, followed by 35 cycles of PCR (denaturation 94°C / 1 min, annealing 49°C BKR1, 52°C BKR2 or 57°C 18S/ 1 min, extension 72°C /1 min) and a final extension of 72°C/ 10 min). PCR products were visualized by agarose gel electrophoresis.

2.7. Statistical analysis

All results depicted represent experiments repeated on at least three separate experiments and using at least three different hearts. Each single condition was performed in octuplicate wells. Data points represent the mean of at least three single experiments ± SEM or the mean of a representative experiment ± SD as indicated. Statistical analysis was performed with SPSS for Mac OS X (SPSS, Inc., Chicago, USA) where necessary. Statistical significance ($p < 0.05$) was established by using non-parametric analysis, i.e., Kruskal-Wallis followed by Mann-Whitney tests.

3. RESULTS

3.1. ACE inhibition and angiogenesis of the heart

3.1.1. ACE inhibition induces angiogenesis of the heart

Initial experiments had shown increased angiogenesis of the rat hearts *in vitro* in ACE-inhibitor perfused hearts (enalapril 10 µM) (experiments in collaboration with PD Dr. Christian Zaugg, Cardiobiology, Department of Research, University Hospital Basel). To investigate whether administration of ACE-inhibitors quinapril and enalapril could enhance angiogenesis *in vitro*, we used an assay of angiogenesis *in vitro* of the heart [27,38]. Pieces of mouse hearts were embedded in fibrin gel and were stimulated every second day with quinapril (1 µg/ml), enalapril (10 µM) and also Bradykinin and angiotensin II to assess if the proangiogenic effect is due to Bradykinin (BK)(10nM accumulation and compare this also with the angiogenic effect of angiotensin II (1 µM).

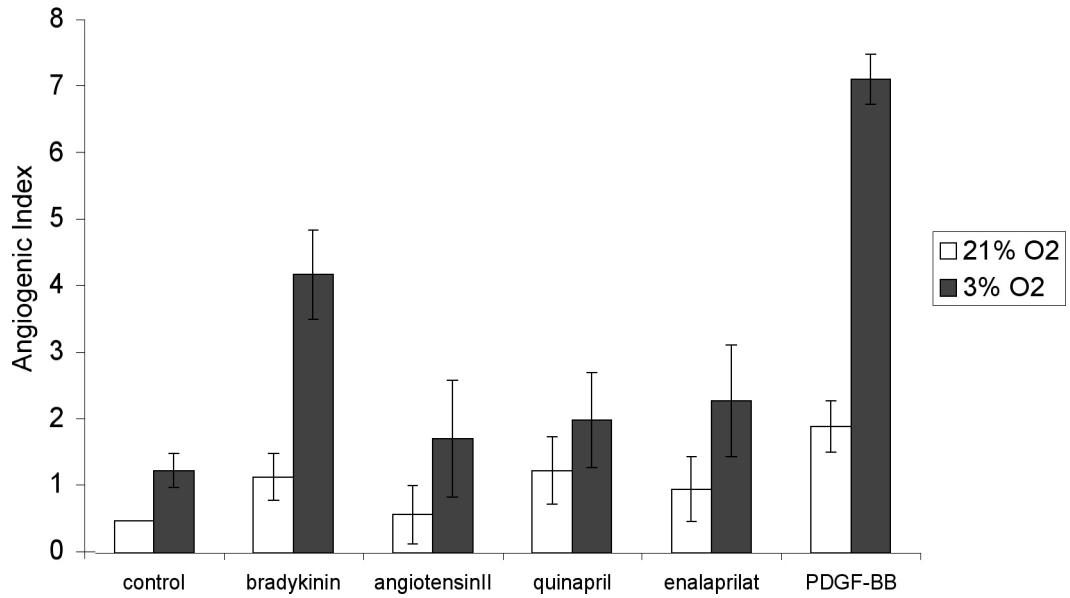


Figure 11. Considerable CLS-formation was observed only under hypoxia (dark columns) but not under normoxia (open columns). Induction of angiogenesis *in vitro* was strongest using 10 nM BK (angiogenic index: 4.4±0.6). 10 µM enalaprilat and 1 µg/ml quinaprilat induced moderate sprout formation (2.3±0.7 and 2.1±0.6 respectively). Angiogenic response of 1 µM angiotensin II was lowest at 1.8±0.7. 10 ng/ml PDGF-BB was used as a positive control (7.3±0.4).

After 10 days of normoxic (21% O_2) and hypoxic (3% O_2) culturing the outgrowth of capillary-like sprouts was evaluated. All of the tested compounds were able to

induce weak angiogenesis under hypoxic condition. Bradykinin however was the strongest in inducing angiogenesis of the rat hearts (Figure 11). This suggested that the proangiogenic effect of ACE-inhibitors under hypoxia might be due to inhibition of Bradykinin-degradation and thus Bradykinin's activity.

3.1.2. Angiogenesis is impaired in left ventricle hypertrophied hearts

In further pilot experiments with hypertrophied versus healthy rat hearts we investigated angiogenesis *in vitro* of rat hearts with left ventricle hypertrophy provoked in response to arterial banding *in vivo* (in collaboration with PD Dr. Christian Zaugg, Cardiobiology, Department of Research, University Hospital Basel). We aimed at assessing whether ACE-inhibitors may favorably affect angiogenesis in the hypertrophied hearts. Both hypertrophied and healthy hearts were embedded in fibrin gels and cultured for 10 days under hypoxia (3%O_2). The pieces of heart were stimulated with 1 µg/ml Quinapril, 10 µM Enalapril, 1 µM Angiotensin II and 10 nM Bradykinin every 2 days. After 10 days outgrowth of capillary-like sprouts, induced by these compounds was evaluated.

ACE inhibitors also induced angiogenesis in these hypoxic hypertrophied and normal hearts. Angiogenesis in the hypertrophied hearts was weaker than in healthy hearts (Figure 12). Thus hypertrophied rodent hearts compared to normal healthy controls display a decreased angiogenic potential. Also Bradykinin was the most potent inducer of angiogenesis.

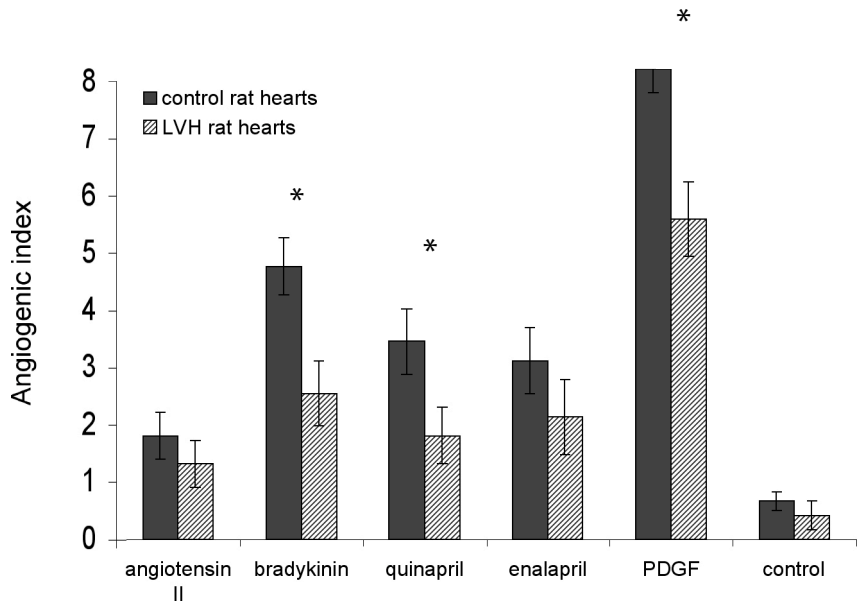


Figure 12. Comparison of angiogenic potential of normal (dark columns) versus left ventricle hypertrophied (striped columns) mice hearts under conditions of hypoxia (3% O_2). Sprout formation in hypertrophied hearts was significantly lower compared to normal mouse hearts when 10 nM BK, 1 µg/ml quinaprilat and 10 ng/ml PDGF-BB were used as angiogenic stimuli (* $p<0.005$, n=3).

3.2. Bradykinin: a potent inducer of angiogenesis

3.2.1. Bradykinin induces angiogenesis under hypoxia

We compared the angiogenic activity of Bradykinin with angiotensin II under hypoxia. Bradykinin induced significant capillary-like sprout (CLS) formation under hypoxia (3% O_2) (angiogenic index of 4.3±0.04, * $p<0.01$, n=3), whereas Bradykinin elicited no significant CLS formation under normoxia (19%-21% O_2) (angiogenic index of 1.8±0.3, n.s. $p>0.01$, n=3) compared to normoxic control (angiogenic index of $0.4\pm0.5_{NRMX}$ and $0.7\pm0.1_{HYPX}$) (Figure 13). CLS formation in response to Bradykinin was similar to that induced by VEGF (7.2±0.02) (Figure 9). Bradykinin was angiogenic only under hypoxia. Hypoxia alone did not induce angiogenesis nor did Bradykinin under normoxia suggesting hypoxia might activate specific hypoxic-sensitive factors in Bradykinin-induced signaling. All further experiments were therefore performed under hypoxia.

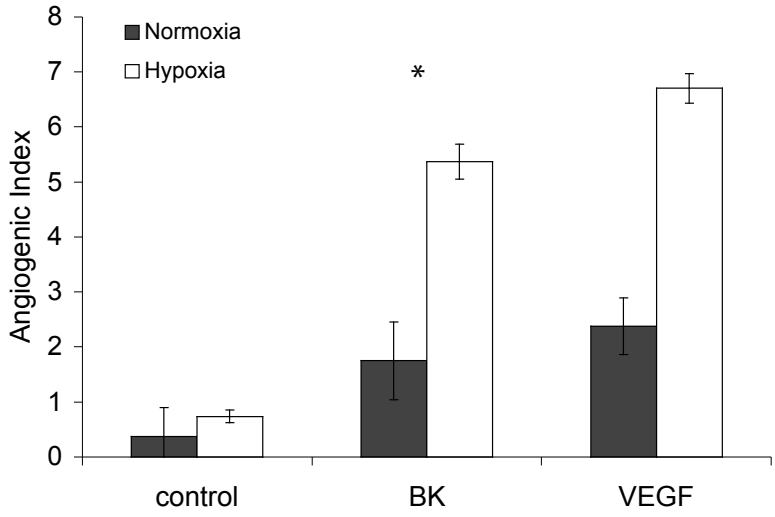


Figure 13. Bradykinin (BK) induces angiogenesis of the mouse heart *in vitro* under hypoxia. Piece of mouse heart were embedded in fibrin gel and exposed to BK (10nM) every 48h for 10 days. Outgrowth of sprouts was observed under hypoxia (3% O_2) and normoxia (21% O_2). VEGF (5 ng/ml) was used as a comparator and positive control. (*=$p<0.05$).

3.2.2. Bradykinin induces angiogenesis in a bimodal way

To determine the effective concentration of Bradykinin required to induce CLS formation, dose-response experiments were performed under hypoxia. Pieces of mouse heart were embedded in fibrin gels and stimulated every 48 hours with increasing doses of Bradykinin. A broad concentration range of Bradykinin from 1 pM to 1 µM was assessed. CLS began to form at 10 pM Bradykinin (angiogenic index of 2.5±0.12), peaking at 10 nM (angiogenic index of 4.7±0.1)(Figure 14). CLS formation was lower at higher concentrations (100 nM Bradykinin) there was a negligible angiogenic response at the highest dose of Bradykinin (1 µM)(angiogenic index of 1.9±0.3).

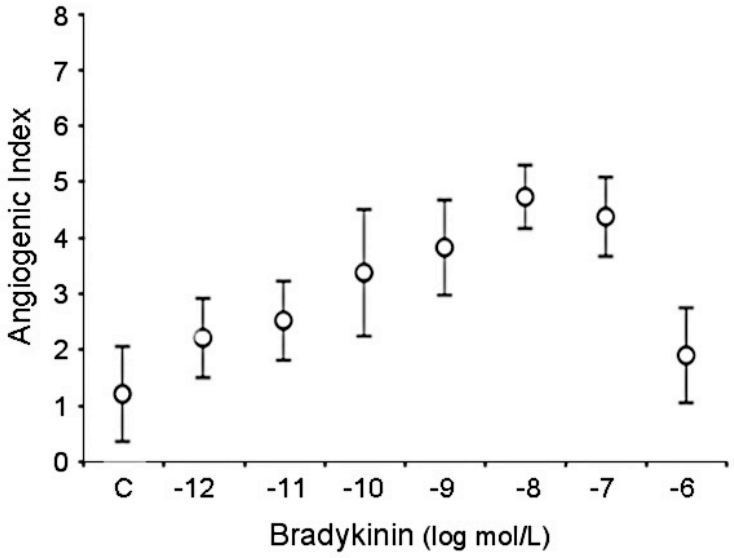


Figure 14. BK induces angiogenesis *in vitro* at low but not at high doses. Pieces of mouse heart were embedded in a fibrin gel and stimulated every 48h with BK (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1000nM) under hypoxia (3% O_2). Sprout formation was observed after 10 days and then evaluated. The angiogenic potency of BK is maximal at a concentration of 10nM (angiogenic index of 4.7±0.5). At higher concentrations, sprout formation does not occur (angiogenic index of 1.9±0.3) similar to non-stimulated control level (angiogenic index of 1.2±0.5).

3.3. Bradykinin receptors and angiogenesis of the heart *in vitro*

3.3.1. Bradykinin receptor expression

To elucidate through which BKR subtype the angiogenic effect of Bradykinin might be mediated, we first examined expression of BKR1 and BKR2 receptors in the heart tissues under hypoxia. BKR1 and BKR2 mRNA levels in mouse hearts were determined by RT-PCR. The heart pieces were incubated under hypoxia or normoxia in the absence or presence of Bradykinin (10nM). RNA was extracted after 2, 4, 12, 24 hours.

Under hypoxia, BKR2 mRNA levels were constitutively expressed and not modulated significantly during the 24-hour period, whereas BKR1 mRNA levels dropped after 2 hours and remained at low levels (Figure 15). We could not detect any significant modulation of BKR1 mRNA levels when comparing either unstimulated with Bradykinin-stimulated heart pieces, or normoxic with hypoxic

heart pieces (Figure 16). BKR2 mRNA levels, however, appeared to be "more stable" during hypoxia (at time points 12 and 24 hours) when compared to the same time points under normoxic culture. These experiments suggest that first, both receptor subtypes are present and could potentially play a role in the Bradykinin-induced angiogenic response and second, they are not upregulated in response to hypoxia. This also means that regulation of Bradykinin receptors does not explain increased angiogenesis in response to Bradykinin during hypoxia.

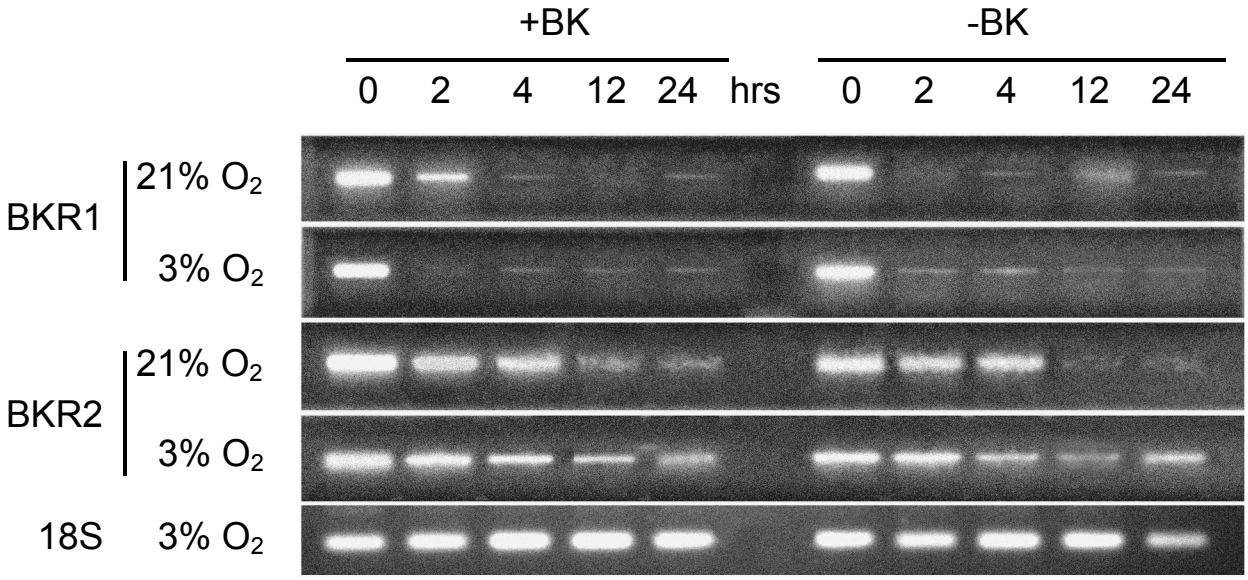


Figure 15. BKR1 and BKR2 mRNA are not upregulated by hypoxia in mouse heart *in vitro*. Pieces of heart were either stimulated with BK (10 nM) or left untreated and incubated under normoxia (21%O_2) and hypoxia (3%O_2). RNA was extracted at the indicated time points and transcribed to cDNA. Transcription of BK1 receptor, BK2 receptor and 18S ribosomal RNA as a control was detected with gene specific primers by PCR.

3.3.2. Bradykinin induces angiogenesis in the heart via BKR2

Bradykinin can act via either of its two receptors, BK1 and BK2 receptors. Several studies suggest different receptor subtypes to be responsible for the angiogenic effect of Bradykinin. In this study we aimed at assessing whether Bradykinin induces angiogenesis of the heart *in vitro* via the BKR1 or BKR2. Therefore pharmacological BKR inhibitors were used. The pieces of heart were

stimulated with Bradykinin (10 nM) under hypoxia. To block the BK2 receptor subtype 100nM of the specific peptide antagonist HOE 140 (D-Arg-[Hyp3, Thi5, D-tic, Oic8]-BK) [105] was used. 10 nM des-(Arg10, Leu9)-Kallidin was used to block the BKR1 [106,107]. Blockade of BKR2 resulted in a potent reduction of Bradykinin-induced CLS formation from heart explants. In contrast, blockade of BKR1 slightly increased CLS formation (Figure 16).

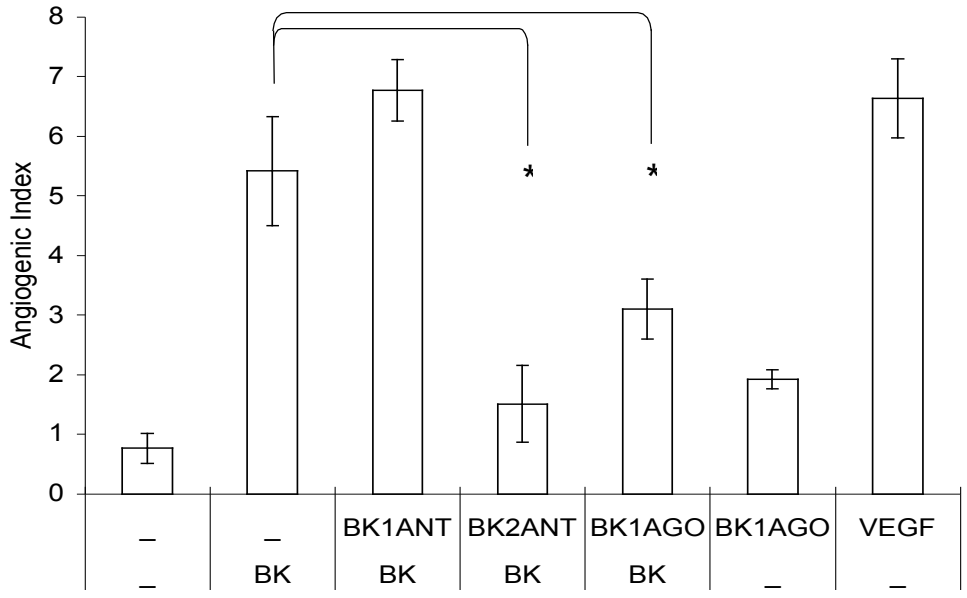


Figure 16. BK induces angiogenesis *in vitro* via the BKR2 under hypoxia. Heart pieces embedded in fibrin gel were stimulated with 10nM BK (angiogenic index of 5.4±0.9). The angiogenic response to low doses of BK was inhibited by HOE 140 (100nM), a specific inhibitor of BKR2 (angiogenic index of 1.55±0.6), but not with an inhibitor of BKR1 (10nM) (lys-des-arg9, leu8)-BK (angiogenic index of 6.8±0.5). Sprout formation was also significantly reduced (angiogenic index of 1.9±0.2) in response to stimulation with a selective BKR1 agonist (LYS- DES- ARG9)- BK (10nM).(*$p<0.05$, n=3).

To further define a potential antiangiogenic role of BKR1, pieces of heart were treated with a selective BKR1 agonist (Lys-Des-Arg9)-BK, (10nM) [108] in the presence and absence of Bradykinin. The BKR1 receptor agonist per se did not mediate angiogenesis, but it significantly reduced Bradykinin-induced

angiogenesis (Figure 16). These data support the hypothesis that the BK1 receptor subtype might have an antiangiogenic role.

3.3.3. Bradykinin-induced angiogenesis is not impaired in BKR1 (-/-) hearts

To further delineate the role of BKR2 we induced CLS formation in BKR1 (-/-) mouse hearts. Neither Bradykinin- nor VEGF-induced CLS formation was significantly inhibited in BKR1 (-/-) mouse hearts (Figure 17). These results provide further evidence that the angiogenic effect of Bradykinin under hypoxia is independent of BKR1 and is mediated via BKR2.

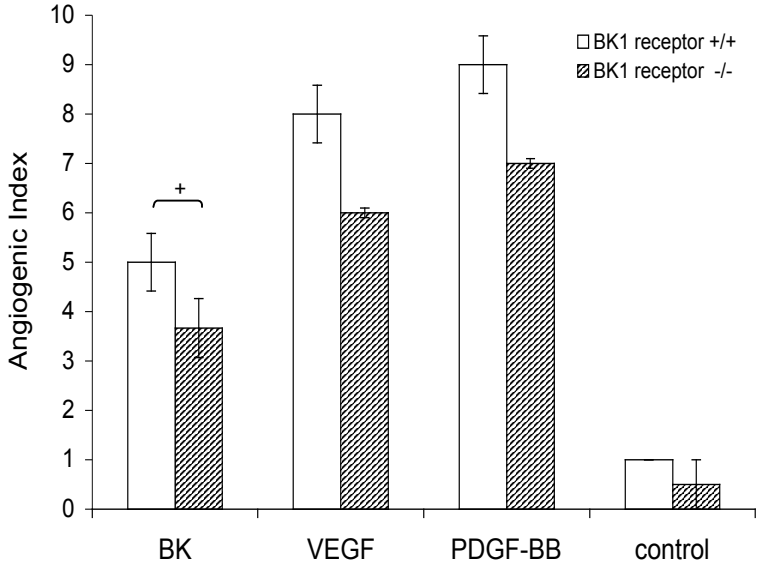


Figure 17. Angiogenesis in response to BK is not significantly affected in BKR1 (-/-) mouse hearts. BKR1 (-/-) mouse hearts and wild-type mouse hearts were stimulated with BK (10nM) every 48 h under hypoxia (3% O_2) and sprout formation was evaluated after 10 days. Angiogenesis was not significantly impaired in hearts of BKR1 (-/-) mice (angiogenic index of 3.7±0.6) in comparison to wild-type heart tissues (angiogenic index of 5.0±0.6). VEGF-induced (5ng/ml) angiogenesis was not significantly impaired in BKR1 (-/-) mice hearts (angiogenic index of 6±0.1) in comparison to CLS formation from the wild-type heart tissues (angiogenic index of 7.9±0.58). += not significant, $p>0.05$, n=3

3.4. Signaling in Bradykinin-induced angiogenesis

3.4.1. Bradykinin-induced angiogenesis depends on nitric oxide biosynthesis

In order to investigate whether Bradykinin-induced angiogenesis via the BKR2 requires NO we used different pharmacological NO-Synthase (NOS) inhibitors during CLS formation. NOS inhibitors were added prior to stimulation of heart pieces with Bradykinin (10nM) or VEGF (5ng/ml). First, unspecific NOS inhibitors L-NAME (Nω-nitro-L-arginine methyl ester, 100μM) and L-NIO (L-N 5-(1-iminoethyl) ornithine, 1μM) were used: Bradykinin-induced sprout formation was significantly reduced by unspecific inhibition of NO biosynthesis (Figure 18). *In vitro* angiogenesis was also impaired in VEGF-treated pieces of heart; however, the abrogation was not as complete as in Bradykinin-induced angiogenesis (Figure 18).

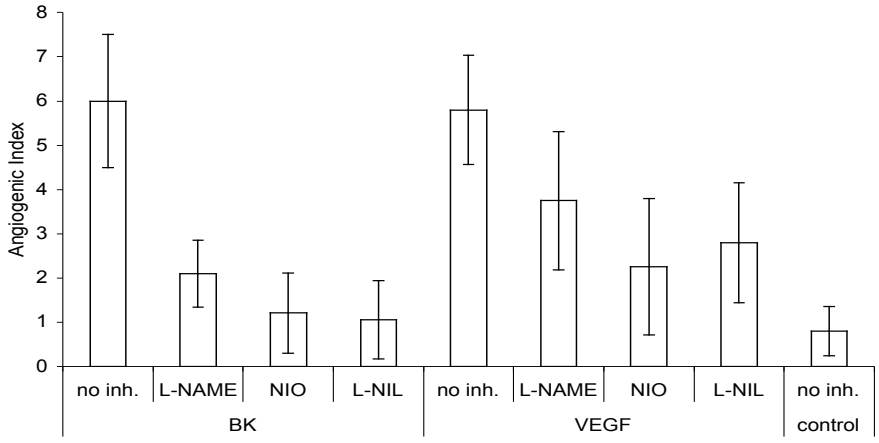


Figure 18. BK-induced angiogenesis depends on NO biosynthesis. Pieces of mouse heart, which had been embedded in fibrin gel, were stimulated with BK (10nM). The angiogenic effect of BK in the mouse hearts under hypoxia (3% O_2) *in vitro* was inhibited by different nitric oxide synthase (NOS) inhibitors; L-NAME 100μM, L-NIL (L-N6- (1-Iminoethyl)-lysine hydrochloride, 10μM, L-NIO (L-N5- (1-Iminoethyl) -ornithine hydrochloride), 1μM. This effect was also observed in VEGF-induced angiogenesis in mouse hearts under hypoxia.

To elucidate whether inducible NOS (iNOS) is required for Bradykinin-induced angiogenesis of the heart under hypoxia, we used L-NIL (N 6 -(1-iminoethyl)-lysine, 10μM), a selective inhibitor of iNOS. Bradykinin-induced angiogenesis was significantly reduced by L-NIL. This effect was also observed in VEGF-induced angiogenesis. Similar to unspecific NO-inhibitors, iNOS inhibition reduced Bradykinin-mediated angiogenesis more potently than VEGF-induced angiogenesis (Figure 18).

3.4.2. Functional iNOS is required for Bradykinin-induced angiogenesis

To further assess the involvement of iNOS under hypoxia, hearts of iNOS-/- mice were used for *in vitro* angiogenesis. In these hearts angiogenesis was stimulated by Bradykinin (10nM) and VEGF (5ng/ml). *In vitro* angiogenesis was completely abrogated in hearts of iNOS-/- mice compared to wild-type mice under hypoxic conditions (Figure 19).

These experiments show that nitric oxide is required for *in vitro* angiogenesis in the heart under hypoxia and that Bradykinin-induced angiogenesis is also dependent on NO biosynthesis. Furthermore, functional iNOS is required for Bradykinin- and VEGF- induced angiogenesis *in vitro* of the heart.

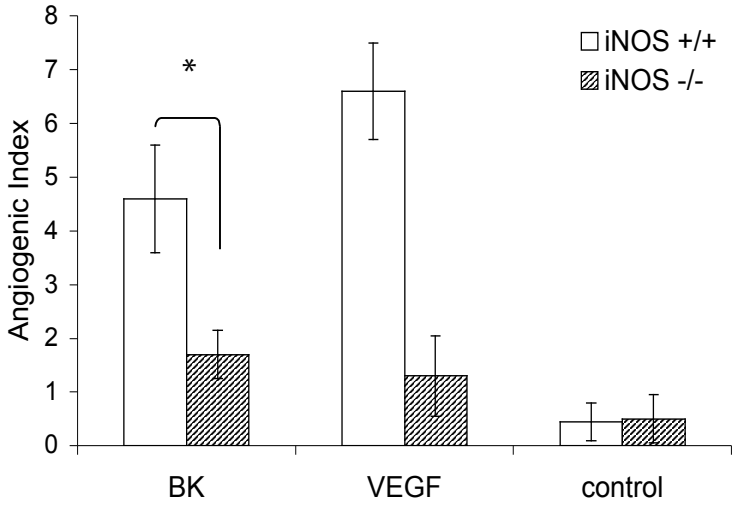


Figure 19. BK-induced angiogenesis requires functional iNOS. iNOS (-/-) and wild-type mouse hearts were stimulated with BK (10nM) every 48 h under hypoxia (3% O_2), and sprout formation was evaluated after 10 days. BK-induced angiogenesis of the heart *in vitro* was almost abrogated in iNOS (-/-) mice (angiogenic index of 1.7±1) in comparison to wt heart tissues (angiogenic index of 4.6±0.4). VEGF-induced (5ng/ml) angiogenesis was also significantly impaired in iNOS (-/-) mice hearts (angiogenic index of 1.3±0.7) in comparison to sprout formation from the wild-type heart tissues (angiogenic index of 6.6±0.9). *; $p<0.05$, n=3.

3.4.3. mTOR-signaling is involved in Bradykinin-induced angiogenesis

These experiments aimed at assessing the further putative molecular mechanisms for Bradykinin-induced angiogenesis under hypoxia. Bradykinin did not show strong angiogenic effects under normoxia, nor did hypoxia induce angiogenesis alone without Bradykinin addition. Thus the role of hypoxia-driven signaling in controlling the angiogenic response to Bradykinin was to be assessed. Pieces of heart were treated with rapamycin in the *in vitro* model of angiogenesis and stimulated with Bradykinin (10nM). The angiogenic effect of Bradykinin was completely abrogated by administering rapamycin (50 nM), an inhibitor of mTOR (mammalian Target Of Rapamycin)(Fig. 20). This experiment shows that Bradykinin induces angiogenesis *in vitro* of the heart via a hypoxia-activated signaling pathway involing mTOR.

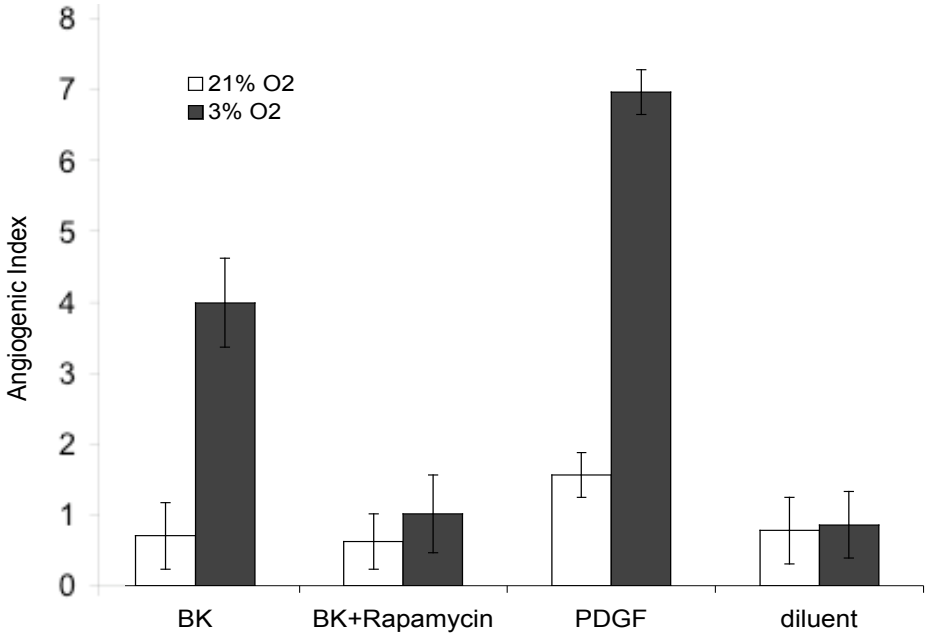


Figure 20. BK (10 nM)-induced angiogenesis *in vitro* under hypoxia (filled column) is strongly reduced (-75%) by administration of rapamycin (50 nM). Normoxic (residual) angiogenesis (open column) in presence of BK was not affected by rapamycin.

3.5. Bradykinin-induced angiogenesis in cancer tissue

We assessed whether it is possible to cultivate cancer tissues in fibrin gels as described for heart explants. Small pieces of melanomas, which were grown in cervical lymph nodes, were taken and embedded in fibrin gels.

As described for the heart explants, cancerous tissue was cultured 10 days and stimulated every 48 hours. After 10 days of incubation under hypoxia sprout formation was evaluated (Figure 21). Quantification of the sprouts was identical to heart.

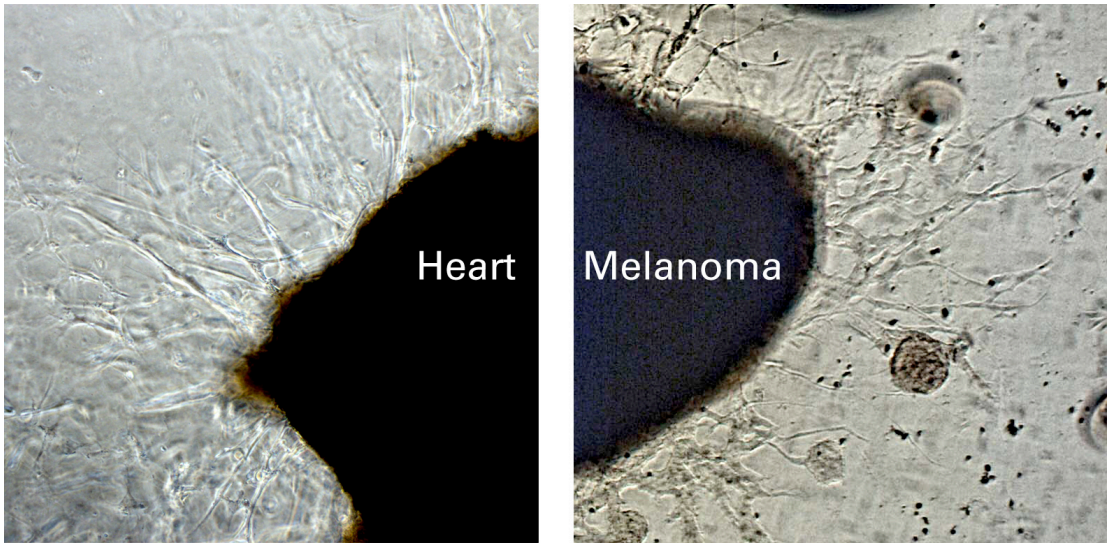


Figure 21. Micrographs taken from pieces of heart (left side) and melanomas (right side) stimulated with 5ng/ml VEGF for 10 days under hypoxia. Note, that capillary sprouts from cancer tissue have a different morphology.

3.5.1. Bradykinin-induced angiogenesis in cancer tissue of wild-type and BKR1 (-/-) mice

We compared Bradykinin-induced angiogenesis in cancer tissues of wild type control mice and in the hearts of the same animals. We observed that both Bradykinin (10nM) and VEGF (5ng/ml) induced angiogenesis in cancer tissues and in the hearts. The angiogenic effect of these two stimuli on the heart (see

page 46, Figure 17) was similar to the results obtained by applying them to cancer tissues (Angiogenic indices of embedded melanomas: Bradykinin (10 nM)=3.9±0.1, VEGF (5 ng/ml)=5.0±0.1, PDGF-BB (10 ng/ml)=6.0±0.3) (Figure 22). In cancer tissues Bradykinin induced more angiogenesis than unstimulated control (Angiogenic Index of diluent=1.3±0.2). However, angiogenesis *in vitro* to VEGF in cancer tissue did not read the same degree as VEGF-induced angiogenesis in the heart (see page 46, Figure 17).

The angiogenic effect of Bradykinin was significantly reduced in cancer tissues of BKR1 (-/-) mice. There was a significant difference between the levels of angiogenesis induced by Bradykinin in cancer tissues of wild type mice and BKR1 (-/-) mice (Angiogenic indices of embedded melanomas: Bradykinin (10 nM)= 1.3±0.1, VEGF (5 ng/ml)= 1.9±0.1, PDGF-BB (10 ng/ml)= 3.4±0.3, diluent= 0.9±0.2). These results suggest that BKR1 is required in capillary-like sprout formation in cancer tissues whereas Bradykinin-induced angiogenesis in heart requires BKR2.

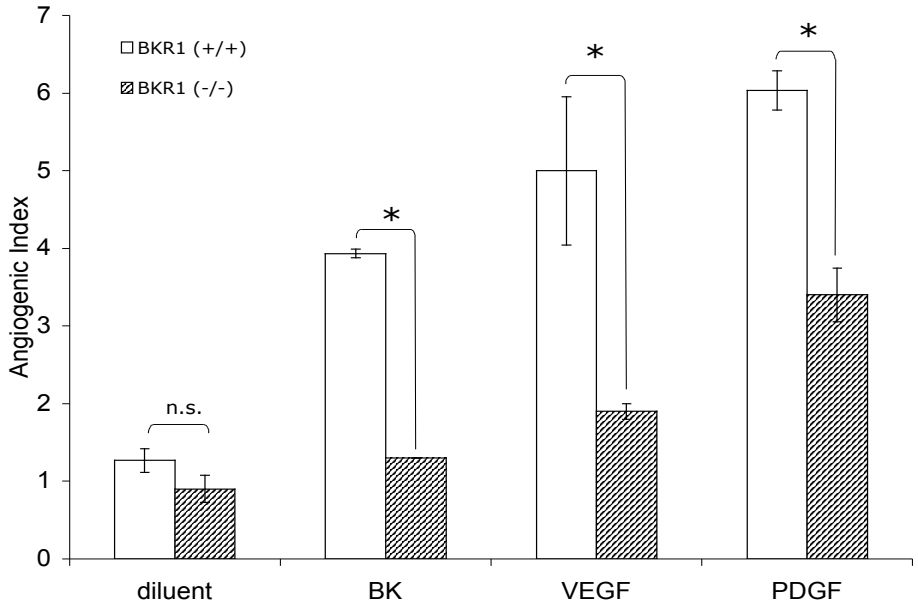


Figure 22. BK- as well as VEGF and PDGF-BB-induced capillary-like sprout formation from melanomas derived from BKR1 (-/-) mice (striped columns) is significantly lower than sprout formation from melanomas derived from wild-type mice (open columns). n.s = $p>0.05$, * = $p<0.05$.

4. DISCUSSION

4.2. Bradykinin-induced angiogenesis in the heart

4.2.1. Angiotensin converting enzyme inhibitors and angiogenesis

ACE-inhibitors are recognized as first-line medications in the hypertension because they are effective in reducing arterial blood pressure and cardiac mass in different animal models and in humans [109,110]. The rationale for using ACE-inhibitors in hypertension is based on the ability to reduce afterload and to a lesser extent, preload, but the effects of these compounds on myocardial structure are not yet fully known. ACE-mediated cardioprotection of ischemic or hypertrophied myocardium might be due to Bradykinin accumulation and its direct cardioprotective action on the myocardium. The actions of myocardium also result in neovascularization of the myocardium, which may contribute to cardioprotection from ischemia.

Several studies had shown previously that administration of ACE-inhibitors increase microvascular density in peripheral tissues and help to reoxygenate affected ischemic tissues. The ACE-inhibitor quinapril enhanced angiogenesis *in vivo* in a rabbit model of hindlimb ischemia [50]. Other ACE-inhibitors have been reported to increase capillary density in rat limb muscle [52,111]. *In vivo* studies in spontaneous hypertensive rats (SHR) showed that vascular growth in the hypertrophied left ventricular myocardium did not keep pace with myocyte hypertrophy, resulting in a decreased capillary density and an increased diffusion distance for oxygen [52,112]. Treatment of these mice with Spirapril, an ACE-inhibitor, increased capillary microvasculature, supporting the concept of a direct action of the drug on myocardium [52].

In our model of angiogenesis of the heart *in vitro* the two ACE-inhibitors enalapril and quinapril both induced the formation of capillary-like sprouts. These and other results also confirmed, that data from *in vivo* studies can be reproduced with our *in vitro* angiogenesis model.

We also evaluated the effects of enalapril and quinapril as well as Bradykinin and Ang II on neovascularization of hypertrophied hearts. LVH is characterized by rarefaction of microvascular density. As a consequence, microvascular rarefaction also contributes to myocardial ischemia. Improvement of the heart performance by increasing capillary density in therapeutic angiogenesis has

emerged as a promising new method of treatment for patients with left ventricular hypertrophy. Our experiments suggest that angiogenesis may be impaired in hypertrophied rat heart tissues in comparison to healthy tissues. Thus, the capacity to form new sprouts was decreased in rats with LVH as compared with rats without LVH. However, Bradykinin not only induced sprout formation in healthy heart tissue, but also in hypertrophied tissue. It was the most potent angiogenic stimulus as compared to enalapril or quinapril, (Bradykinin > enalapril = quinapril > AngII).

Microvascular rarefaction in LVH may explain our observation of decreased angiogenesis of hypertrophied hearts *in vitro*: Since angiogenesis is defined by the growth of new microvessels from pre-existing ones, the decreased capillary density provides less capillary 'resources' for this process to set on. Alternatively, endothelial dysfunction associated with and all major cardiovascular factors may contribute to impaired angiogenesis. Dysfunctional endothelial cells have a decreased capacity to produce nitric oxide, to proliferate and migrate and a decreased expression of matrix-metalloproteinases (MMPs). Endothelial migration, proliferation and matrix degradation are crucial steps in angiogenesis (see introduction). Thus, endothelial dysfunction, especially decreased NO-biosynthesis (see below) may contribute to impaired angiogenesis.

4.2.2. Mechanism of Bradykinin-induced angiogenesis

In our studies Bradykinin showed its potent angiogenic effect only under conditions of hypoxia. Furthermore, Bradykinin-induced angiogenesis occurred at a low concentration range; at low nanomolar doses, Bradykinin was a potent inducer of new sprouts- comparable to VEGF- in our model of angiogenesis *in vitro*. At high concentrations, Bradykinin did not induce angiogenesis. Furthermore, the BKR2 antagonist and, surprisingly, a BKR1 agonist blocked Bradykinin-induced angiogenesis. Thus, the Bradykinin-induced response in angiogenesis is bimodal. Similarly bimodal Bradykinin responses have been shown on afferent arterioles of rabbits *in vivo*, where Bradykinin induced vasodilation at low concentrations and vasoconstriction at higher concentrations[113].

Our results show that Bradykinin induces capillary-like sprout formation of mouse heart explants under hypoxia via BKR2 activation. BKR2 blockade by HOE 140 strongly reduced Bradykinin-induced capillary-like sprout formation. Additionally, inhibition of BKR1 by Lys-(des-Arg^9, Leu^8)-BK does not affect Bradykinin-induced capillary sprout formation. Inhibition of BKR1 tended even to increase angiogenesis *in vitro*. Thus, no pro-angiogenic role could be identified for the BKR1. In contrast, when BKR1 was stimulated by the BKR1 specific agonist (des-Arg^9)-BK, angiogenesis was strongly reduced in the presence of 10 nM Bradykinin, suggesting an anti-angiogenic role for the BKR1. Such an anti-angiogenic role for the BKR1 has not been described previously. Myocardial ischemia is reported to upregulate BKR2 in male Sprague Dawley rat hearts [114]. In BKR2 knock out mice, the capability of ACE inhibition to induce angiogenesis is abrogated [38]. Confusingly, other reports suggest that the BKR1 is required for the angiogenic response to Bradykinin [77,115]. Still, in our study, Bradykinin-induced angiogenesis was not impaired in BKR1 -/- mice. Thus, no pro-angiogenic role could be identified for the BKR1. Our results in hearts derived from BKR1 -/- mice, support our results obtained with the pharmacological agonists and antagonists for BKR1 and BKR2.

These opposing roles of the two Bradykinin receptor subtypes may potentially explain the bimodal angiogenic response to Bradykinin, i.e., induction of angiogenesis only at low nanomolar but not at high concentrations. There are no common mechanisms explaining bimodal dose responses. Mostly interacting receptor pathways are involved [116]. Hypothetically, the presently described bimodal effect may be due to the interplay of pro-angiogenic effects via the BKR2 at low concentrations and anti-angiogenic effects via BKR1 at high concentrations. This assumption is in accordance with the differing affinities of the two receptors for Bradykinin [117]. BKR2 has a high affinity (K_D = 0.19 nM) and BKR1 has a low affinity (K_D = 29000 nM) [118] for Bradykinin. An additional or alternative mechanism for the bimodal response might be a rapid de-sensitization and internalization of BKR2 in response to high BK concentrations [98,119,120].

Bradykinin elicits many of the intracellular signaling responses that are typically associated with the activation of growth factors, hence also angiogenic growth factors [27,63].

At the level of mRNA, we have detected both receptor subtypes. We also observed a rapid and sustained decrease of BKR1 mRNA both under normoxia and hypoxia with or without Bradykinin. Our result suggests an artefactual downregulation of BKR1 expression when taking the myocardium from its normal environment. However, when we used BKR1 agonist, the angiogenic effect of Bradykinin was reduced significantly, suggesting BKR1 playing an active role in Bradykinin-induced angiogenesis *in vitro* of the heart. However, none of the receptors were consistently upregulated under hypoxia as detected by semiquantitative RT-PCR. *In vivo* expression of BKR1 may be differently regulated than *in vitro*. In ischemic skeletal muscle BKR1 was reported to be upregulated [38].

Other groups were also confronted with artifacts regarding the role of BKR1 while examining BKR2 (-/-) mice. The normally nonexpressed BKR1 gene becomes expressed in BKR2 (-/-) mice and may assume the function of BKR2 [121]. Discrepancies may thus be due to different experimental settings or the type of Bradykinin receptor knockout animals used.

Further studies in hearts from BKR1 -/- and BKR2 -/- will be necessary to assess the different requirement of BKR subtypes during Bradykinin-induced angiogenesis.

4.2.3. Signaling in Bradykinin-induced angiogenesis *in vitro*

Role of NO. Different experimental and clinical observations support a molecular and biochemical link between vasodilation, NO production and angiogenesis. Angiogenesis *in vivo* is accompanied by vasodilation of preexisting capillaries. The potent vasodilator properties of Bradykinin are due to its ability to increase NO synthesis [50,122]. NO is a key regulator of hypoxia-/ischemia-induced angiogenesis [27,38,123]. Bradykinin was shown to increase eNOS expression specifically via BKR2 in a model of surgically induced hindlimb ischemia in mice [51] and in cardiac capillary endothelial cells [78]. Inhibition of the NOS-pathway by pharmacological NOS inhibitors abrogates BKR1-mediated endothelial cell proliferation [77,123]. Thus, different NO synthases activated via distinct Bradykinin receptor subtypes may increase NO and promote angiogenesis.

In the present study we provide further evidence for a link between NO production and angiogenesis, since NOS inhibitors markedly reduced Bradykinin-

induced angiogenesis of the heart under hypoxia. This reduction was almost total with iNOS inhibitors. Moreover, Bradykinin-induced sprout formation was almost completely abrogated in hearts from iNOS-deficient mice. As a result Bradykinin-induced angiogenesis depends on the production of NO and requires functional iNOS.

Interestingly, brief episodes of myocardial ischemia and reperfusion (ischemic preconditioning) elicit a biphasic response in NOS activity *in vivo*, whereby there is an immediate activation of eNOS followed by delayed upregulation of iNOS [124]. Furthermore, with prolonged hypoxia, increased expression and activity of iNOS and thus elevated NO biosynthesis leads to downregulation of eNOS expression [124,125]. These findings *in vivo* are consistent with our observations of an iNOS-dependence for Bradykinin-stimulated angiogenesis in the heart. Antidromic activations of different NOS isoforms may explain some of the discrepant findings attributing angiogenic functions to either BKR2 [51,73,74,126] or BKR1 [77,115]. BKR1 may be proangiogenic in an eNOS-dependent and acute manner [77,124]. However, as shown in this study Bradykinin-induced angiogenesis over a prolonged period of hypoxia occurs via BKR2 and in an iNOS-dependent manner.

Role of mTOR. A potent angiogenic effect of Bradykinin was only observed under conditions of moderate hypoxia (3% O_2). Rapamycin, the inhibitor of mammalian target of rapamycin (mTOR) effectively inhibited Bradykinin-induced angiogenesis under conditions of hypoxia. mTOR is a central modulator of cell growth and a prime target for anti-cancer drugs in therapeutic development. mTOR plays a critical role in transducing proliferative signals mediated through the phosphoinositol 3 kinase (PI3K)/ protein kinase B signaling pathway. Recent studies and our own previous data suggest that mTOR, which is the rapamycin target protein, functions as a positive regulator of hypoxia and hypoxia-inducible factor-1 (HIF-1) [143, 144-]. mTOR-dependent angiogenesis under hypoxia is mediated at least in part by binding of HIF-1 to the hypoxia response elements in the promoter region of VEGF, bFGF and iNOS genes, thus enhancing their expression [127,128].

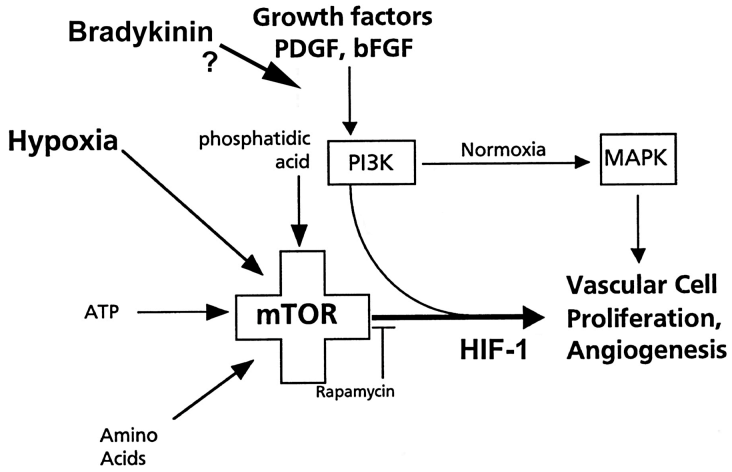


Figure 23. mTOR mediated signaling under conditions of hypoxia. Bradykinin signaling may be amplified through hypoxic activation via mTOR.

By targeting mTOR, the antiproliferative agent rapamycin also inhibits signals required for cell cycle progression, cell growth and proliferation. Rapamycin has recently shown promising anti-tumor activities, which are attributed to the inhibition of mTOR. Our findings demonstrate mTOR to be involved in hypoxia-facilitated angiogenesis of the heart *in vitro* in response to Bradykinin (Figure 23). This suggest that anticancer activities of Rapamycin may also base on its antiangiogenic effects during hypoxia.

Thus, hypoxia significantly modulates intracellular signaling and expression of transcription factors that enhance the angiogenic response induced by various effector molecules generated upon ACE-inhibition such as Bradykinin.

4.3. Bradykinin-induced angiogenesis in cancer

In situ carcinomas remain dormant and undetected for many years, and metastases are rarely associated with these small (2-3 mm^3) vascular tumors. Yet, several months or years later, an *in situ* tumor may switch to the angiogenic phenotype, induce the formation of new capillaries, and start to invade the surrounding tissue. The "angiogenic switch" depends on a net balance of positive and negative angiogenic factors in the tumor. Thus, the angiogenic phenotype may result from the production of growth factors, such as FGF-2 and VEGF, by tumor cells and/or the down-regulation of negative modulators in tissues with a quiescent vasculature[17].

Bradykinin is an important growth factor in cancer since certain Bradykinin antagonists could show remarkable anti-cancer activities[79]. It is suggested that Bradykinin increases vascular permeability in ascitic tumors and promotes tumor growth by increasing angiogenesis. Enhanced vascular permeability is the universal and hallmark event meditated by Bradykinin that is shared in cancerous tissues as well as inflammatory tissues [18,129].

Thus, a role for Bradykinin in tumor biology has been suggested in several reports, but its precise roles and the molecular basis for its actions have not yet been elucidated. Its action remains speculative despite many studies in animal and humans. Angiogenesis is reported to be regulated in an organ-specific way suggesting a need for organ-specific models [130].

4.3.1. Bradykinin-induced angiogenesis in melanomas: a BKR1-mediated response ?

We assessed angiogenesis of melanoma tissue grown in the lymph nodes of wild-type and BKR1 (-/-) mice and compared it to angiogenesis of wild-type and BKR1 (-/-) mice hearts. Bradykinin induced capillary sprout formation in melanoma tissue under conditions of hypoxia in wild-type animals. However, Bradykinin induced much less capillary sprout formation in melanomas gained from BKR1 (-/-) mice. This is surprising, since capillary sprouts readily formed in hearts from BKR1 (-/-) mice, i.e., angiogenesis was not impaired in BKR1 (-/-) mice hearts. This suggests, that vascularization of melanomas is regulated differently than in the heart and requires BKR1.

Furthermore, our results have confirmed, that organ-specific assays may yield very interesting and varying results that cannot be reproduced by conventional assays [130].

4.3.2. Vascularization of melanomas: a BKR1 mediated inflammatory response?

Several reports describe the induction of the BKR1 by inflammatory and infectious stimuli in rats[131,59]. In a study with BKR1 (-/-) mice a low response to inflammatory stimuli was observed[131,132]. All these facts suggest the importance of modulated molecules including the inducible BKR1 in body defense mechanisms. However, there are no reports yet on the significance of BKR1 induction in relation to gastrointestinal disorders or carcinoma, [133]

During inflammatory processes BKR1 expression increases via a NF-κB-transcrition factor [134]. Melanoma progression requires increased vascularization[135]. In a very recent report, NF-kB is has been associated with the vascular progression of melanoma [135]. Thus, we hypothesize that melanoma progression may depend on an inflammatory response initiated via a NF-kB-dependent induction of BKR1.

Angiogenesis of melanomas should also be assessed using BKR2 (-/-) mice as well as BKR agonist and antagonists. In nude mouse xenotransplants of lung and prostate cancers the Bradykinin antagonists inhibit angiogenesis and activation of membrane metalloproteases (MMP 2 and 9)[79]. In the xenotransplants certain Bradykinin antagonists showed higher potency than standard anti-cancer drugs, without evident toxicity to the hosts [79]. Results with these peptides suggest that a new generation of anti-inflammatory and anti-cancer drugs may be at hand [79].

5. CONCLUSIONS AND OUTLOOK

Components of the Renin-angiotensin-aldosteron-system and pharmacological agents to suppress it have also been found to control angiogenesis *in vitro* and *in vivo*. A key enzyme of the Renin-Angiotensin-Aldosteron-System is the Angiotensin-Converting-Enzyme (ACE). ACE inhibitors, which are broadly used in clinical medicine for the treatment of hypertension and congested heart failure, block the conversion of Angiotensin I to Angiotensin II and inhibit the breakdown of Bradykinin, a potent vasodilator and mediator of inflammation. Vasodilation precedes angiogenesis and is also mechanistically linked to it. In line with this, Bradykinin can induce angiogenesis. Therefore the angiogenic response of ACE inhibitors in rat hind limbs may be mediated via accumulation of Bradykinin.

Using an *in vitro* assay validated by reproducing known *in vivo* phenomena, we have investigated the role of Bradykinin in inducing angiogenesis of the heart *in vitro.* Furthermore, we have better delineated the subtype of Bradykinin receptors and Bradykinin-induced signaling that are required for angiogenesis.

Under condition of moderate hypoxia (3% O_2) Bradykinin induced capillary-like endothelial sprout formation at low concentrations but not under normoxia (21% O_2). Using a variety of techniques (RT-PCR, functional assays using agonists and antagonists, knock out animals, protein detection assays) we have demonstrated that Bradykinin acts via the Bradykinin receptor 2 to induce angiogenesis *in vitro* in the heart. Furthermore, Bradykinin-induced angiogenesis of the heart requires nitric oxide biosynthesis (using pharmacological inhibitors) and inducible nitric oxides synthase (using iNOS knockout mice). Furthermore, we found that angiogenesis of the heart might be different from that seen in tumors (melanomas). In a mouse melanoma cancer model in BKR1 (-/-) mice angiogenesis *in vitro* was significantly reduced as compared to wild-type control animals. This suggests that melanomas, in contrast to hearts, require functional BKR1 to develop new microvessels.

Taken together, this thesis delineates mechanisms by which Bradykinin induces angiogenesis in the hypoxic heart. These results were further compared with angiogenesis in a mouse cancer model. The results suggest that Bradykinin plays an important role in angiogenesis of the heart and that angiogenesis in the heart may be mediated via different Bradykinin receptors from that in melanomas in response to Bradykinin (Figure 24, page 62).

Future investigations may further delineate the potentially anti-angiogenic role of the BKR1 in heart angiogenesis, as well as the role of BKR2 in melanoma vascularization. Additionally, the pro-angiogenic role of BKR1 will have to be assessed in other tumor types (Figure 24).

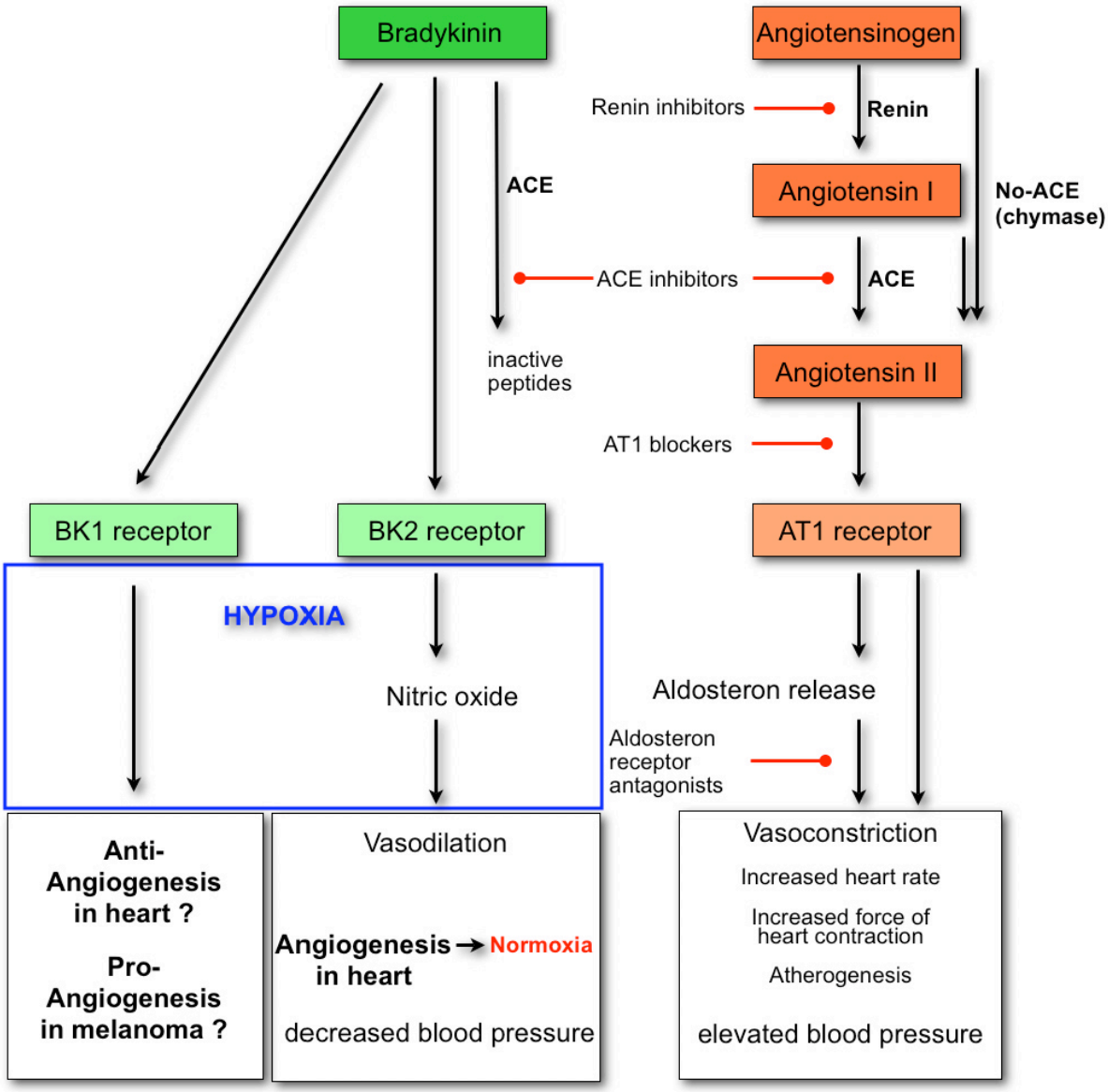


Figure 24. Visualization of keypoints presented in this thesis. Question marks indicate potential issues to be resolved in the future.

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8. CURRICULUM VITAE

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PERSONAL DATA

Date of Birth:	23.08.1969
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Address:	Grether str 19
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EDUCATION

2000-2004	**PhD-Thesis** Laboratory of Vascular Biology, Dept. of Research of the University Hospital Basel, Switzerland with Prof. Dr. Edouard Battegay, Head of Medical Outpatient Department, Head of the Hypertension Clinic, University Hospital Basel and Prof. T. W. Güntert, Head of Non-clinical Research Department, Hoffmann La-Roche Ltd. The Thesis deals with the role of the Renin-Angiotensin-Aldosteron-System (RAAS) and Bradykinin in Angiogenesis of the Heart.
1992-1999	**Master of Science in Pharmacy,** Eberhard-Karls-University of Tübingen/Germany, concentration in Pharmacology, Pharmaceutical Chemistry, Pharmaceutical technology and Pharmaceutical Biology
1997	1st Staatsexamen
1998	2nd Staatsexamen
1999	3rd Staatsexamen
1991-1992	Study of Biology at the Friedrich-Wilhelm-University of Bonn, Bonn/Germany
1991	German Abitur in Medical Sciences
1989-1991	Studienkolleg für ausländische Studenten (College for foreign Students)
1988-1989	Study of English Language at the University of Tehran

SPECIAL COURSES & WORKSHOPS

2/2003	International Workshop on New therapeutic Targets in Vascular Biology.
2/2002	Courses in Pharma-Business (Pharma R&D, Patents and Licensing) at the Pharmacenter of the Swiss Federal Institute of Technology Zurich.
11/1998	Grundlagen der Arzneimittelzulassung, (Basics of Drug Regulatory Affairs).

WORK EXPERIENCE

2/2000–5/2001	Hoffmann-La Roche Ltd, Basel/Switzerland, Research Department for Cardiovascular and Metabolic Diseases under Dr. Gianni Gromo. Identification of new pharmacological concepts and characterization of new target molecules and molecular mechanisms.
5/1999–10/1999	Unterlinden Apotheke, Freiburg/Germany. Pharmacist trainee.
11/1998–4/1999	Hoffmann La-Roche AG, Grenzach-Wyhlen/Germany, Practical Training in the drug Regulatory Affairs Department and in the Professional Services Department of Dr. Lazlo Bethlen's group. Activities included: Updating of national scientific information according to Core Data Sheet. Preparations of Variations for the local Health Authorities. Preparation of a Database for questions most frequently asked by health care professionals.
1994-1997	Part-time work as shop manager in a Benetton Shop Tübingen/Germany.

PUBLICATIONS

1. Development of an Assay of Angiogenesis of the Heart (2002), Kiefer FN, Dieterle T, Humar R, **Neysari S**, Li W, Battegay EJ, Journal of vascular Res., 39(S1): 64 (PAN 17).

2. BK induces Heart Angiogenesis via BKR2 and NO-Biosynthesis under Hypoxia (8/2002). **Neysari S**, Kiefer FN, Humar R, Battegay EJ, Journal of Vascular Research. 39(S1): 65 (PAN 21).

3. Hypertension and Angiogenesis (2003), Kiefer FN, **Neysari S**, Humar R, Li W, Munk VC, Battegay EJ. Curr Pharm Des.; 9(21): 1733-44.

4. Bimodal, iNOS-dependent induction of Angiogenesis by BK via different BK receptor subtypes in hypoxic Mouse Hearts . (Submitted). **Neysari S**, Humar R, Kiefer FN, Zaugg Ch& Edouard Battegay.

PRESENTATIONS

Poster presentation at 22nd Meeting of the European Society for Microcirculation, Aug. 2002, Exeter, Devon/UK. BK induces Heart Angiogenesis via BKR2 and NO-Biosynthesis under hypoxic conditions.

Oral presentation at the 8th Cardiovascular Biology and Clinical Implications Meeting, Oct. 2002, Villars/Switzerland. BK induces at low but not at high doses Angiogenesis of the Heart under conditions of Hypoxia.

LANGUAGES

German:	excellent
English:	excellent
Spanish:	basic knowledge
Persian (Farsi):	mother tongue
Turkish:	fluent

NOTES