Angiogenesis *in vitro* of the healthy mouse heart under hypoxia: The role of Angiotensin II and Nitric Oxide

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To my family for helping me be myself at all times

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SUMMARY

Angiogenesis is the process by which blood microvessels are formed from existing ones. Angiogenesis is required for development. It is also important for reducing myocardial hypoxia due to coronary and ischemic heart disease; in myocardial infarction or chronic ischemic heart disease angiogenesis responds to tissue hypoxia by new vessel formation (angiogenesis), which diminishes myocardial ischemia. However, physiological angiogenesis is usually insufficient to re-establish an adequate blood supply to the myocardium, which decreases its proper functioning. Therapeutic angiogenesis in the heart aims at increasing new vessel formation in ischemic myocardium and thus improving myocardial function by increasing blood flow (oxygen and nutrient supply). This may contribute to preventing heart failure and sudden cardiac death. Unfortunately no assay is available to investigate questions around angiogenesis in an easy format and in a way that does not use big numbers of animals.

Angiogenesis and hypertension are intrinsically linked; angiogenesis is impaired in hypertension, and microvascular rarefaction is a mainstay of hypertension-induced target organ damage. Many metabolic pathways, for example the Renin-Angiotensin-Aldosterone-System (RAAS) or Nitric Oxide (NO), are involved in the development of hypertension, hypertension-induced target organ damage and also angiogenesis. Contrariwise, treatment of hypertension by drugs such as ACE-inhibitors not only reduces blood pressure and hypertension-induced target organ damage but also improves angiogenesis and thus tissue oxygenation. Specifically, accumulation of Bradykinin in response to ACE inhibition may result in angiogenesis. A study in our laboratory lead us to conclude that impaired angiogenesis in hypertension may result from impaired NO biosynthesis and not from elevated blood pressure itself. In addition, activation or RAAS or other factors may affect angiogenesis in hypertension.

The general aim of this thesis was to first contribute at developing a new angiogenesis assay of the heart *in vitro* and to then use it to investigate the role of Angiotensin II and Nitric Oxide on angiogenesis in the heart *in vitro*, independent of blood pressure. We also aimed at understanding mechanisms involved in these responses.

In order to further study questions of angiogenesis and hypertension in a relevant target organ, we developed and validated a new *in vitro* assay for investigating angiogenesis of the heart. At the time most experiments were being performed *in vivo* since no appropriate *in vitro* model was available. *In vivo* experiments require a large number of animals, are difficult to perform and are often associated with pain to the animals and their death. Our new *in vitro* model solved or reduced some of these problems. We found that both hypoxia and serum (5%) are required for angiogenesis to occur in the adult mouse heart *in vitro*. We analyzed the morphology of the different sprouts and found they were always composed by endothelial cells, and that smooth muscle cells or pericytes align along the sprouts. We conclude that angiogenesis of the heart *in vitro* can be investigated with a simple assay that allows a large series of experiments to be carried out in a relatively short time and with a minimum number of animals. We have shown that our model is suitable to investigate the actions of different substances on angiogenesis of the heart, i.e., both substances that induce angiogenesis and those that may inhibit it.

Subsequently we used our newly developed assay to investigate the role of iNOS on angiogenesis of the mouse heart and aortae under hypoxia. We found that the heart is more sensitive to the different inhibitors than aortae. *In vitro* angiogenesis of the heart in iNOS knock out mice, in hypoxia, was totally absent. We therefore concluded that organ specific pathways must exist for angiogenesis; and that for angiogenesis of the mouse heart, in hypoxia, iNOS is essential.

In the last part of the thesis we describe the work done to understand the role of Angiotensin II in the hypoxic mouse heart. By using different pharmacological agonists and antagonists as well as knock out animals we were able to conclude that the AT2 receptor is the one responsible for angiogenesis in response to Angiotensin II in the healthy and hypoxic mouse heart in our *in vitro* model. Further experiments led us to conclude that the angiogenic effect of Angiotensin II via the AT2 receptor in the hypoxic mouse heart is mediated via a mechanism that involves the Bradykinin receptor 2.

In conclusion we have developed a new *in vitro* model of angiogenesis *in vitro* of the heart. Using this model we have analyzed angiogenesis of the hypoxic mouse heart and then characterized effects of Ang II and NO on angiogenesis *in vitro*.

1. INTRODUCTION

1.1 Angiogenesis

1.1.1 Introduction

Angiogenesis is the process by which blood microvessels are formed from existing ones. Angiogenesis is a common process occurring during development as well as during ischemic heart disease and tumor growth [1, 2]. A capillary is formed by the tunica intima, which typically only consists of the endothelium, its basal lamina and an incomplete layer of cells surrounding the capillary, the pericytes. Pericytes, serve to stabilize the forming capillary tube, have contractile properties and can regulate blood flow in capillaries [3]. Angiogenesis involves a series of steps that can be seen represented in figure 1. First, blood vessels dilate and pericytes detach allowing endothelial cells (EC) to migrate into the perivascular space following angiogenic stimuli; they will then adhere to each other and create a lumen and finally these new vessels will fuse with others creating new circulation [4]. Each of these steps is highly regulated and controlled by a variety of mechanisms and molecules.

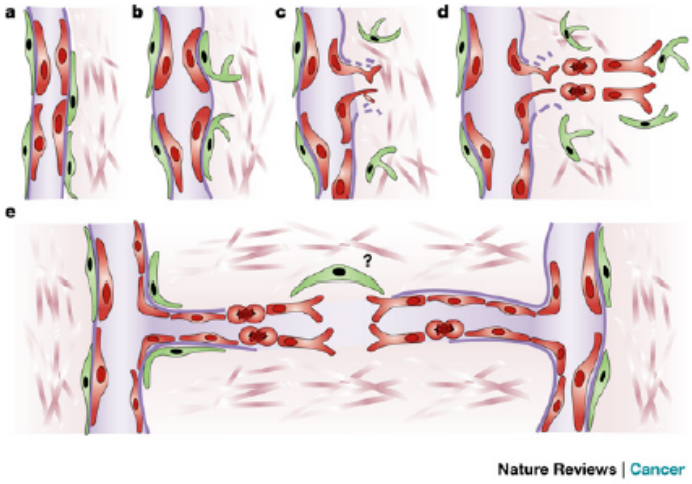

Figure 1*:* New blood vessel formation (a) Blood vessels arise from pre-existing capillaries or post-capillary venules. (b) First, pericytes (green) detach and blood vessels dilate before the basement membrane and cellular matrix are degraded. (c) This allows endothelial cells (red) to migrate into the perivascular space towards angiogenic stimuli. (d) Endothelial cells proliferate, loosely following each other, and presumably guided by pericytes and extracellular matrix. (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-vessels sprouts will fuse with other sprouts to build new circulatory systems. Little is known about the fusion mechanism. Figure by Bergers.2003. [4].

1.1.2 Angiogenesis of the heart

In diseased hearts such as those that suffer from myocardial infarction or ischemic heart disease, angiogenesis is a very important process. In these conditions the blood vessels supplying blood to the heart muscle narrow or harden, reducing the supply of oxygen and nutrients to the heart musculature, which is essential for it's proper functioning. This may eventually result in a portion of the heart being suddenly deprived of its blood supply (ischemia) leading to the death of that area of heart tissue, resulting in a heart attack (myocardial infarction). An improvement in collateral circulation and thus of blood flow helps enhance the function of diseased hearts by delivering oxygen and nutrients to areas where needed. Two important stimuli in these conditions are inflammation and hypoxia [5]. Inflammation and presence of inflammatory cells is known to be sufficient to induce the formation of new microvessels [6-8]. Hypoxic areas of the heart lead to the release of angiogenic growth factors and nitric oxide (NO) [9]. Both hypoxia and myocardial ischemia upregulate the expression of angiogenic factors [10]. This naturally occurring angiogenesis is not enough to fully compensate for the loss of oxygen in ischemic heart conditions, in consequence new ways to increase blood supply are being investigated by potentation of physiological angiogenesis.

1.1.3 Therapeutic Angiogenesis

Therapeutic angiogenesis involves the development of collateral blood vessels supplying ischemic tissues, either endogenously or in response to administered growth factors to improve circulation in affected areas. Clinical trials have been conducted with administration of growth factors to the human heart [11-14]. Most studies have focused on the use of known angiogenic factors such as Fibroblast Growth Factor (FGF) and Vascular Endothelial Growth Factor (VEGF). Different methods have been tried to deliver the angiogenic molecules as a natural protein or by gene transfer but to date no optimal method has been found [10, 15-17]. Therefore the search continues for the best way to induce therapeutic angiogenesis. Currently, treatment using stem cells is being tested; this therapy may be the preferred therapeutic choice because of its clinical applicability and regenerative capacity. Different types of stem cells such as those leading to the generation of, bone marrow cells, skeletal and smooth muscle cells, vascular endothelial cells, mesothelial cells, adipose tissue stroma cells, dental stem cells, and embryonic and fetal cells, have

been proposed for regenerative medicine. To date ethical as well as immunological and technical reasons make their clinical use limited [18].

1.2 Hypoxia and the Hypoxic Heart

1.2.1 Defining Hypoxia

The air we breathe contains 21% oxygen and cells in our body are exposed to an oxygen concentration ranging form 0.5% to 12% depending of the tissue and organ. In circulating arterial blood oxygen concentration is about 14% and less than 10% in the myocardium [19]. These variations make it difficult to define hypoxia. A practical definition is that hypoxia designs any state in which oxygen delivery does not meet the demand of an organ, tissue or cell [20]. In the myocardium oxygen concentrations between 0.5-3% are described as hypoxia [19]. For experimental cell culture, hypoxia is defined as 0.5-3% oxygen concentration. At this level toxicity and growth-inhibition of cells is not present, and cellular responses to hypoxia can be triggered [20, 21]. Therefore, we used oxygen concentrations within this range.

1.2.2 The Hypoxic Heart

When a mayor coronary artery is occluded or when deficient growth of the microvasculature cannot keep up with the rate of the hypertrophying myocardium, the myocardium becomes ischemic and the heart is said to be hypoxic [22]. At this stage the vascular supply cannot keep up with the metabolic demands of the heart and this leads to a state of hypoxia [23]. If the oxygen supply is permanently blocked this can lead to myocardial infarction.

Hypoxia will stimulate proliferation of both smooth muscle cells (SMC) and endothelial cells (EC) favouring the creation of new vessels to restore oxygenation. Hypoxia upregulates various important genes and signalling pathways that have to do with these processes [21, 24]. Hypoxia-induced genes are controlled by transcription factor Hypoxia Inducible Factor 1 (HIF-1). HIF-1 activates genes responsible for angiogenesis such as VEGF [25], nitric oxide synthases [26] and many more. Under normal oxygen conditions HIF-1 is rapidly degraded. Under hypoxia HIF-1 is stabilized by inhibition of a prolyl hydroxylase, thus preventing HIF's proteolitic degradation.

1.3 Angiotensin II and Bradykinin

1.3.1 The Discovery of Angiotensin II

Around 1958 two groups of investigators came to the conclusion that ischemia-linked hypertension they were observing was produced by an enzymatic chain of events involving renin, in which a pressor substance is generated. The group of Braun-Menendez, in Argentina, named it 'hypertensina' [27] and the group of Page, in the USA, called it 'angiotonin' [28]. By collective agreement the hybrid name Angiotensin was defined. Angiotensin II (Ang II) is an octapeptide that is generated by the cleavage from angiotensinogen through the action of two different peptidases (Angiotensin Converting Enzyme (ACE) and renin). Ang II exerts a wide range of physiological actions on the cardiovascular, renal and endocrine systems. Its main action is the regulation of blood pressure and the maintenance of fluid homeostasis through the renin- angiotensin-aldosteron system (RAAS) [29].

Ang II has a variety of effects on the body; it is a potent vasoconstrictor throughout the body. In the kidneys, it constricts glomerular arterioles, this increases arteriolar resistance, raising systemic arterial blood pressure and decreasing the blood flow. However, the kidneys must continue to filter enough blood despite this drop in blood flow. To keep glomerular blood pressure up Ang II also constricts efferent arterioles, which forces blood to build up in the glomerulus, increasing glomerular pressure. The glomerular filtration rate is thus maintained, and blood filtration can continue despite lowered overall kidney blood flow. However, increased glomerular filtration pressure damages the glomerulus with time. Ang II also acts on the adrenal cortex causing the release of aldosterone, which acts on the tubules (i.e., the distal convoluted tubules and the cortical collecting ducts) in the kidneys, causing them to reabsorb more sodium and therefore water from the urine. This also increases blood pressure due to an increase in intravascular volume. Aldosterone also acts on the central nervous system to increase a person's appetite for salt, and to make them feel thirsty. These effects directly act to increase the amount of fluid in the blood, making up for a potential loss in volume and/or to increase blood pressure [29].

1.3.2 Synthesis of Angiotensin II

Ang II is synthesised by a cascade of enzymatic reactions [30] as depicted in figure 2. In the first step, angiotensinogen is cleaved by renin to form Angiotensin I (Ang I), which is biologically inactive. The Angiotensin Converting Enzyme (ACE) then converts Ang I into the active octapeptide Ang II by removing 2 amino acids. The sequence of Ang II in mammals is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe. Ang II is then further metabolized to form Ang III and Ang IV which in turn are further metabolized into small amino acid fragments [31, 32].

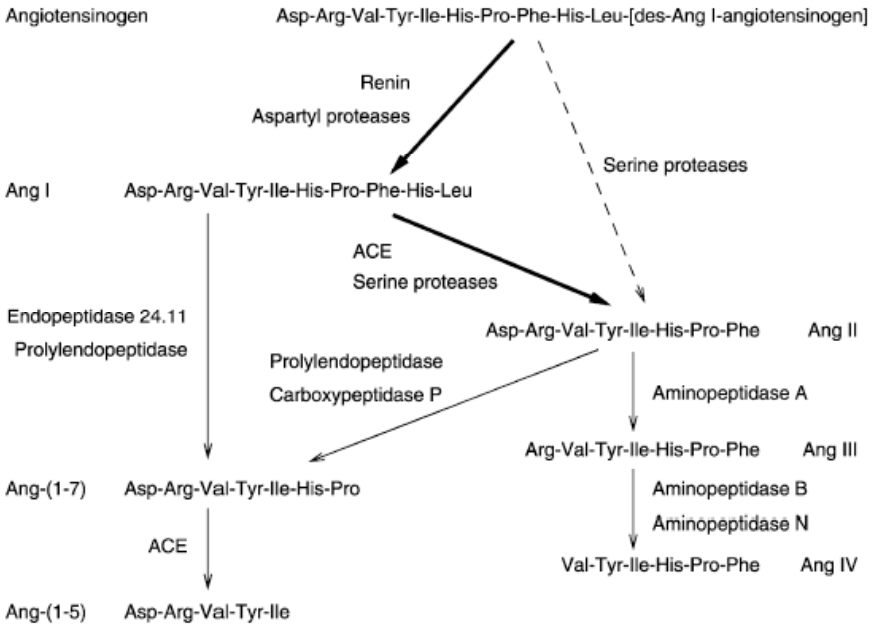

Figure 2*:* Outline of the renin-angiotensin system. ACE: angiotensin converting enzyme. Of the several alternate pathways of metabolism, the bold, plain and dashed arrows indicate pathways of decreasing contribution, although minor pathways can always assume major importance when the main pathway is inhibited. Figure by DJ Campbel, 2003 [33].

1.33 Angiotensin II Receptors

Ang II exerts its actions by binding to one of its two major receptors, AT1 or AT2.

AT1 Receptor

The AT1 receptor is responsible for most of the known physiological actions of Ang II in the peripheral tissue and brain [34]. AT1 receptor was successfully cloned in 1991, in humans it's localized on chromosome 3 and contains 359 amino acids [35, 36]. This sequence is 95% identical to that of rat and bovine AT1 receptors [37, 38]. In rats AT1a and AT1b receptors subtypes exist. They are highly homologous, 95% in regard to amino acid sequence and 92% in regard to nucleic acids [34]. The AT1 receptor is a seven transmembrane domain receptor and belongs to the family of G-protein-coupled receptors (GPCR).

AT1 receptors are expressed in all tissues where Ang II is known to exert its classical effects, in vascular smooth muscle cells (VSMC), uterus, kidneys, heart, lung, urinary tract and lungs [39-44]. The expression and quantity of AT1 receptors varies among these different tissues. The pattern of distribution suggests that this receptor exerts very important biological functions in these tissues; distribution of expression can be affected by various pathological conditions such as hypertension and myocardial infarction [45-47].

The signalling mechanisms of this receptor have been extensively studied. As a member of the G-Protein Coupled Receptor (GPCR) family it seems to couple mainly to G_q activating a cascade that involves Phospholipase C (PLC) leading to the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). These second messengers are then responsible for several of the effects regulated by the AT1 receptors such as vascular smooth contraction and secretion of aldosterone [34]. Other signalling pathways also exist and are represented in figure 3.

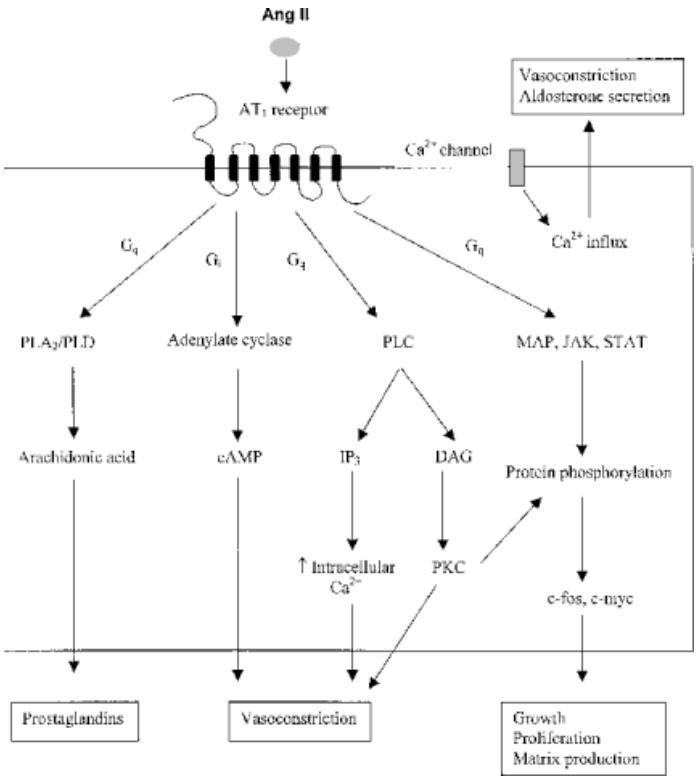

Figure 3*:* Signal transduction mechanisms and physiological effects mediated by AT1 receptor**.** Figure by DT Dinh et al 2001 [48]

Deletion of the gene encoding the AT1A receptor subtype in mice significantly reduces blood pressure and pressor responses to infused Ang II [49, 50]. Conversely, in AT1B receptor knock-out mice, systemic blood pressure is normal, suggesting that the AT1A receptor is the major receptor involved in blood pressure regulation [51]. However, exogenous Ang II infusion can still elicit pressor effects in AT1A deficient mice, which can be blocked by AT1 receptor antagonists [52]. Although these pressor responses were smaller than those seen in wild-type mice, it still suggests a role for the AT1B receptor in blood pressure regulation, particularly in the absence of functional AT1A receptors. Recently, transgenic mice over-expressing the AT1 receptor in cardiac myocytes have been shown to develop cardiac hypertrophy and remodelling, with no change in blood pressure, and died prematurely of heart failure [43]. This suggests that Ang II, via activation of AT1 receptors, is directly involved in the development of cardiac hypertrophy and heart failure, independently of blood pressure. In 1998 AT1A/AT1B mice were generated by Oliverio et al [53]. These mice present diminished growth, vascular thickening of the kidney and atrophy of the inner renal medulla [53]. These mice have no systemic pressor response to infusions of Ang II, and have reduced blood pressure [53]. Taken together all their results point

to the important of AT1 receptors in mediating the physiological functions of the RAAS.

AT2 Receptor

The AT2 receptor has been cloned in mouse, rat and humans [54-58] in all species it resides as a single copy on the X-chromosome [59]. The cDNA encodes for a receptor with 363 amino acids, with high homology on the nucleic acid (89%) and amino acid levels (91%) between rodent and humans. Comparison with the AT1 receptor yields a very poor homology, of just 34%.

AT2 receptors are predominantly expressed in fetal tissues. After birth the ratio of AT1 to AT2 receptors is reversed, with the AT1 being the dominant one in most tissues of the adult organism [60]. In the adult both receptors can be found in the adrenal gland, vascular endothelial cells, kidneys and heart, whereas the AT2 receptor predominates in the uterus, ovarian granulosa cells and some brain areas [60-62]. Even though the AT2 receptor is not commonly expressed in adult tissues it can dramatically increase under pathological conditions such as vascular injury, congestive heart failure, renal failure, brain ischemia and myocardial infarction [60]. This expression patterns can be influenced by a variety of growth factors and hormones in different tissues [63].

The signalling pathway of the AT2 receptor has turned out to be quite complex, even though the AT2 receptor has the structural features of a seven transmembrane receptor, it does not reveal the shared functional features of this family of receptors. The pathways that have been so far described seem to depend on tissues or cells as well as on the experimental conditions. Some of the known pathways are described in figure 4. One important pathway is the activation of guanylate cyclase leading to increased levels of cGMP, NO production and its release [64-66].

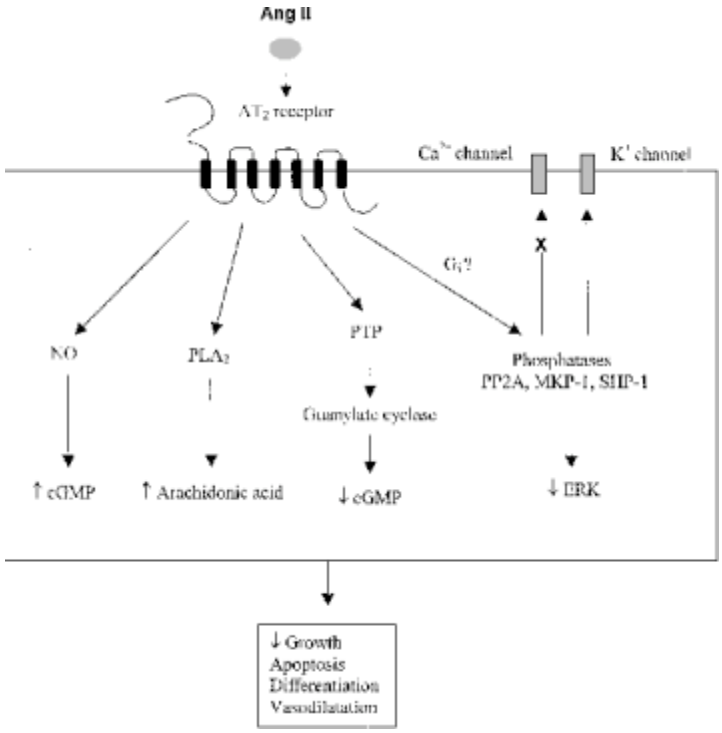

Figure 4*:* Signal transduction mechanisms and physiological effects mediated by AT2 receptor**.** Figure by DT Dinh et al 2001 [48]

In contrast to AT1 receptor gene deletion, targeted deletion of the AT2 receptor gene in mice results in animals that develop normally, but have an impaired drinking response to water deprivation as well as a reduction in spontaneous movements. Their baseline blood pressure is normal, but they show an increased vasopressor response to injection of Ang II, [67, 68]. This suggests that the AT2 receptor mediates a vasodilation effect and may functionally oppose the effects mediated by the AT1 receptor, possibly via bradykinin and NO [69].

Given that AT2 receptors are highly abundant in fetal tissues, such as the heart, kidney and brain, and disappear soon after birth, they were believed to play an important role in foetal development. However, AT2 receptor knockout mice apparently develop and grow normally and do not show observable morphological defects, suggesting that the AT2 receptor may not be essential for fetal development [67, 68]. AT2 receptor knockout mice also show impaired drinking responses to water deprivation and reduced exploratory behaviour [67, 68].

1.3.4 Agonists and Antagonists of the Angiotensin II Receptors

Selective AT1 antagonists abound, they all belong to the family of so called 'sartan' compounds and are currently used to effectively treat hypertension [60]. In this work we have used Losartan as an AT1 inhibitor. A selective AT2 antagonist is PD 123319, which binds with high affinity to this receptor. An agonist for AT2 receptor is also being currently used in research, CGP 42112; no specific AT1 receptor agonist has been described to date.

1.3.5 The Kallikrein-Kinin System and Bradykinin

Bradykinin (BK) belongs to the family of kinins, molecules that participate in inflammatory processes by activating endothelial cells. They also lead to vasodilation, increased vascular permeability and NO production [70, 71]. BK is liberated by plasma kallikrein from high molecular weight kininogen (see figure 5) and acts as a potent short-lived vasodilator.

The BK receptors, BKR1 and BKR2, belong to the family of GPCRs. BKR2 is predominant in most tissues and constitutively expressed [72] and is the one responsible for most of the known biological effects.

The Bradykinin receptor 2 knock-out mouse is a very good model to study the interaction of BK and Ang II. These mice develop mild hypertension, cardiac hypertrophy, chamber dilation and myocardial damage. They have elevated left ventricular and end-diastolic pressure, and show exaggerated vasopressor response to Ang II. In the presence of Ang II they have increased blood pressure and reduced renal blood flow [73, 74].

1.3.6 The interaction between the plasma Kallikrein-Kinin system and the Renin Angiotensin system.

The plasma kallikrein-kinin system (KKS) has been traditionally linked to physiological homeostasis. In recent years the link between BK and NO production and NO mediated pathological effects has been increasingly studied [75]. The endothelial cell-associated active kallikrein cleaves the higher molecular weight kininogen to form BK, which in turn regulates vascular tone by stimulating NO production in endothelial cells via BKR2 [76].

ACE and prolycarboxypeptidase (PRCP), are key molecules linking both systems, as can be seen in figure 5. ACE is responsible for converting Ang I to the

vasoconstrictor Ang II but it also degrades BK into smaller peptides. PRCP degrades Ang II into $Ang_{(1-7)}$ which, causes vasodilation and stimulates NO and prostaglandin formation. This potentiates the effects of BK. PRCP can also convert prekallikrein into kallikrein that then cleaves kininogen to liberate BK. The KKS is activated by various stimuli such as tissue damage, ischemia and inflammation [77].

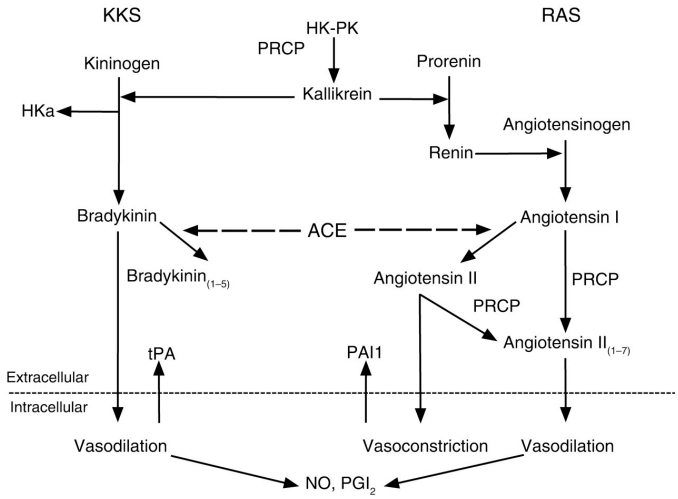

Figure 5: The interaction between the plasma KKS and RAS. Plasma kallikrein converts prorenin to renin, and renin has the ability to convert angiotensinogen to angiotensin I. Angiotensin-converting enzyme (ACE) converts inactive angiotensin I to the vasoconstrictor angiotensin II. Angiotensin II stimulates plasminogen activator inhibitor 1 (PAI1) release from endothelial cells. At the same time ACE degrades bradykinin into bradykinin (1–7) (not shown) or bradykinin (1–5), a peptide with thrombin inhibitory activity. PRCP is the enzyme that degrades angiotensin II or angiotensin I to the vasodilating peptide, angiotensin II (1–7). Angiotensin II (1–7) stimulates NO and PGI 2formation, which potentiates the effects of bradykinin. PRCP also has the ability to convert PK to kallikrein. Formed kallikrein digests kininogens to liberate bradykinin, leaving a kinin-free kininogen (HKa) that has anti-proliferative and anti-angiogenic properties. Thus, PRCP, the same enzyme that degrades the vasoconstrictor angiotensin II, leads to the increased formation of the vasodilators bradykinin and angiotensin II (1–7). Finally, the resulting bradykinin stimulates TPA, NO, and PGI 2 formation, thus counterbalancing the prothrombotic effect of angiotensin II. Figure by Schmaier, 2002. [76]

1.4 Nitric Oxide

1.4.1 Introduction to Nitric Oxide

Nitric Oxide is a gas and a free radical first described in the 1980s as an endothelium-derived relaxant of vascular smooth muscle cells [78, 79]. NO relaxes the smooth muscle in the walls of the arterioles. At each systole, the endothelial cells that line the blood vessels release NO. This diffuses into the underlying smooth muscle cells

causing them to relax and thus permit the surge of blood to pass through easily. Since its discovery NO has been extensively studied, discovering its important role as a signalling molecule in most biological systems. Many of its effects are mediated through the activation of guanylyl cyclase which increases 3',5'-cyclic guanosine monophosphate (cGMP) formation from guanosine 5'-triphosphate (GTP) [80-83].
NO is synthesized by a group of enzymes called nitric oxide synthases (NOS) [80-85]. Three isoforms have been identified, neuronal NOS (nNOS), inducible or inflammatory NOS (iNOS) and endothelial NOS (eNOS). These forms share approximately 50% homology of sequence and have nearly identical mechanisms of action. NOSs catalyze the production of NO and citrulline from L-arginine, oxygen and NADPH-derived electrons. All these NOSs are found in different cellular locations and can be induced by different stimuli even if only iNOS is called inducible NOS [84].

1.4.2 iNOS

In normal healthy quiescent cells iNOS is not expressed [85]. But after induction by immunologic or inflammatory stimuli iNOS is expressed in a large variety of cells [85-88]. Cytokines such as interferon-gamma (IFN), tumor necrosis factor (TNF), interleukin-1 and -2, and lipopolysaccarides (LPS) cause an increase in iNOS mRNA, protein, and activity levels. Once expressed, iNOS generates large volumes of NO often reaching detrimental levels [84, 85, 87]. NO is induced in various pathological conditions such as inflammatory and non-inflammatory pain, asthma, arthritis, septic shock and in the brain after trauma or ischemia [85, 87]. Newer data point also to beneficial effects of iNOS induction, as observed in iNOS knockout animals. iNOS is important in skin wound healing and healing of the intestinal mucosa as well as required for bone resorption by osteoclasts. Importantly it is also involved in angiogenesis [85, 87].

1.4.3 Angiogenesis and Nitric Oxide

NO is required for *in vivo* angiogenesis and for endothelial tube formation *in vitro* [89-91]. NO causes vasodilation, a key process that precedes endothelial sprout formation at the start of the angiogenic process [92, 93]. NO is also involved in angiogenesis by influencing the activity of various growth factors that are known to be angiogenic such as VEGF [1, 5, 94]. The importance of NO for angiogenesis has

also been seen in knockout mice, both eNOS and iNOS knockouts display impaired angiogenesis in models of capillary in-growth into Matrigel plugs and models of skin flap survival respectively. [87, 95-97].

NO is also required for the process of wound healing of which angiogenesis is a key process [98-100]. In tumours it has been shown that NO is essential for growth [93, 101-104]. In a model of arterial hypertension impaired angiogenesis was observed. This is thought to be brought about by an impaired NO biosynthesis [105]. Exactly how NO affects angiogenesis is still unclear, most of the signalling mechanisms remain to be studied [106, 107].

1.5 Hypertension

1.5.1 Arterial Hypertension and microvascular rarefaction

Blood pressure is a measurement of the force applied to the walls of the arteries as the heart pumps blood through the body. The pressure is determined by the force and volume of blood pumped and the size and flexibility of the arteries. Blood pressure is continually changing depending on activity, temperature, diet, emotional state, posture, physical state, and medication use. Normal blood pressures fall in the range of 90/60 mm Hg to 130/80 mm Hg. The first of these 2 numbers is the systolic blood pressure reading, and it represents the maximum pressure exerted when the heart contracts. The second number is the diastolic blood pressure reading, and it represents the pressure in the arteries when the heart is at rest [29].

Arterial hypertension is the elevation of blood pressure beyond the normal range. Persistent hypertension is one of the risk factors for stroke, heart attack and heart failure as well as of chronic renal failure. Arterial hypertension is also characterized by reduced NO biosynthesis, increased vasoconstriction and by microvascular rarefaction (reduction of microvascular density). Rarefaction has been observed in animal models of hypertension and also in human patients [108, 109]. Microvascular rarefaction contributes to increased peripheral resistance and in consequence to the development of chronic arterial hypertension [108], it also impairs blood flow to important areas of the heart such as the myocardium [109]. Hypertension causes damage to target organs, leading to high levels of morbidity and mortality. Untreated

hypertension can lead to heart diseases such as left ventricular hypertrophy and myocardial ischemia that may lead to stroke and sudden cardiac death. [110].

1.5.2 Hypertension and the heart

Over time hypertension and its associated risk factors may lead to the development of left ventricular hypertrophy (LVH) or myocardial infarction as schematically shown in figure 7. LVH is characterized by rarefaction of microvessels and in consequence ischemia of the left ventricle. This can lead to systolic and diastolic dysfunction which can lead to further heart failure.

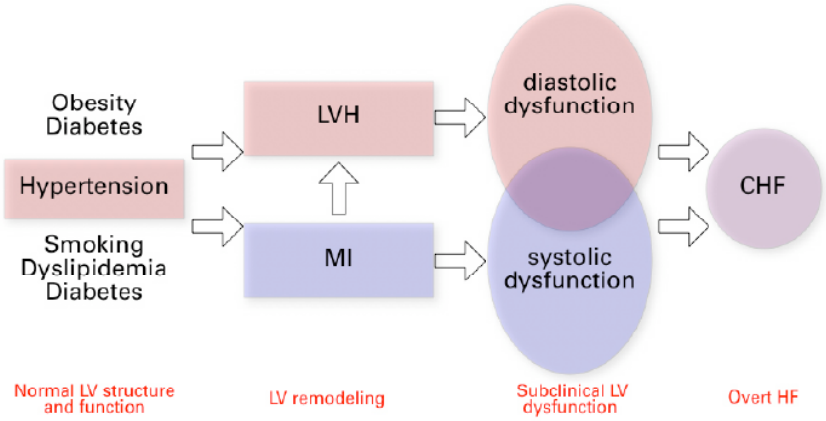

Figure 7: Progression from hypertension to heart failure. CHF= congestive heart failure; CV=Cardiovascular; HF= heart failure; LV= left ventricular; LVH= left ventricular hypertrophy; MI= myocardial infarction. Figure by R. Humar

1.5.3 Anti-Hypertensive Drugs

The goal of antihypertensive therapy is to normalize blood pressure and to prevent cardiovascular complications of hypertension, such as heart failure, stroke, end stage renal disease, and death.

Many treatments for hypertension are currently on the market but two of the antihypertensive drugs relevant to this thesis and most commonly used are ACE inhibitors and Ang II receptor blockers (ARB). The beneficial effects of ACE inhibitors are generally attributed to a decrease in the ACE mediated generation of Ang II and the accumulation of bradykinin [111]. ARB's act by selectively blocking the binding of Ang II to the AT1 receptor but not to the AT2 receptor [112-114]. Clinical evidence suggests that this mechanism of action has some benefits that go beyond blood pressure control. Patients after acute myocardial infarction and patients with chronic heart failure benefit from treatment with ARB equally compared to

treatment with ACE inhibitors. Hypertensive patients with electrocardiographically left ventricular hypertrophy treated with ARB seem to have an additional benefit in terms of morbidity and mortality compared to treatment with beta-blockers [115]. In the early stages of stroke, patients treated with ARB have a lower 12-mounth mortality than patients receiving placebo [116].

1.5.4 Hypertension and Angiogenesis

As mentioned above microvascular rarefaction has been identified in hypertensive patients and animal models [1, 2]. Late-onset hypertension is associated with a lack of coronary angiogenesis [117]. Inadequate angiogenesis is closely related to hypertension per se or hypertension related organ damage [1, 2]. Studies suggest that the impaired angiogenesis observed may be due to reduced NO biosynthesis, activation of the RAAS and other factors [105, 118]. Thus, improved angiogenesis could help relieve the onset of hypertension and prevent organ damage.

1.6 References

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2. RATIONALE AND AIMS

Angiogenesis is a highly regulated process that arises in response to hypoxia and other stimuli to relieve tissue ischemia. To study this process most experiments are performed *in vivo*, requiring large number of animals. These experiments are often associated with pain and death of many animals. An appropriate *in vitro* model would be desirable to solve some of these problems.

Nitric oxide (NO) is a key signaling molecule and regulator of angiogenesis. NO produced by inducible NOS (iNOS) modifies both angiogenesis and vascular permeability. iNOS expression is significantly increased during myocardial ischemia and infarction. This lead to the idea of studying whether a deficiency of iNOS impairs myocardial angiogenesis.

Therapeutic angiogenesis is based upon improving myocardial function by increasing blood flow (oxygen and nutrient supply) to ischemic areas of the heart, thus preventing heart failure and sudden cardiac death. After renal ischemia Ang II restores blood flow by stimulating the development of collateral circulation, independent of its hypertensive effect [1]. This lead to the idea of studying Ang II as a possible target for therapeutic angiogenesis.

The aim of this study was to investigate the role of Angiotensin II and Nitric Oxide on angiogenesis in the heart *in vitro*, independent of blood pressure, using our newly developed system of angiogenesis *in vitro* of the heart. We also aimed to understand the mechanisms involved in these responses.

Our objectives were:

1. To investigate and validate a model of angiogenesis of the heart *in vitro*
2. To characterize the cells involved in the sprouting
3. To assess the response of the heart to important angiogenic stimuli under different experimental conditions
4. To study the function of iNOS on angiogenesis of the heart and aorta *in vitro* under conditions of hypoxia

5. To determine the angiogenic effect of Ang II *in vitro* in normoxia and hypoxia.
6. To study the receptors involved in the angiogenic response due to Ang II in hypoxia.
7. To study the involvement of the Bradykinin pathway in the angiogenic response elicited by Ang II

Reference

[1] Fernandez LA, Caride VJ, Twickler J, Galardy RE. Renin-angiotensin and development of collateral circulation after renal ischemia. Am J Physiol. 1982; 243: H869-75.

3. PUBLISHED WORK- RESULTS

The results I obtained during my PhD thesis have been published or submitted to publishing as described below.

3.1
Hypertension and angiogenesis.
Kiefer FN, Neysari S, Humar R, Li W, Munk VC, Battegay EJ.
Curr Pharm Des. 2003;9(21):1733-44.

3.2
A versatile *in vitro* assay for investigating angiogenesis of the heart.
Kiefer FN, Munk VC, Humar R, Dieterle T, Landmann L, Battegay EJ.
Exp Cell Res. 2004 Nov 1;300(2):272-82.

3.3
iNOS is required for *in vitro* angiogenesis of hypoxic healthy mouse hearts.
Munk VC, Humar R, Kiefer FN, Battegay EJ.
Submitted.

3.4
Angiotensin II induces angiogenesis *in vitro* through an AT2 – BKR2 pathway in the hypoxic mouse heart.
Munk VC, Sanchez de Miguel ML, Humar R, Butz N, Eriksson U, Hein L and Battegay EJ.
Submission pending approval by Merck Sharp and Dohme-Chibret AG

Hypertension and Angiogenesis

F. N. Kiefer, S. Neysari, R. Humar, W. Li, V. C. Munk

and E. J. Battegay.

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Abstract

Arterial Hypertension (AH) is characterized by reduced nitric oxide (NO) biosynthesis, activation of the Renin-Angiotensin-Aldosteron-System (RAAS), vasoconstriction, and microvascular rarefaction. The latter contributes to target organ damage, especially in left ventricular hypertrophy, and may partially be due to impaired angiogenesis.

Angiogenesis, the formation of new microvessels and microvascular networks from existing ones, is a highly regulated process that arises in response to hypoxia and other stimuli and that relieves tissue ischemia. In AH, angiogenesis seems impaired. However, blood pressure alone does not affect angiogenesis, and microvascular rarefaction is present in normotensive persons with a family history for AH.

Normal or increased NO in several processes and diseases enables or enhances angiogenesis (e.g. in portal hypertension) and reduced NO biosynthesis (for example, in a rat model of AH, in other disease models *in vivo*, and in endothelial NO Synthase knock out mice) impairs angiogenesis. Angiogenic growth factors such as Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) induce NO and require NO to elicit an effect. Effector molecules and corresponding receptors of the RAAS either induce (Bradykinin, Ang II) or perhaps inhibit angiogenesis. The pattern of Bradykinin- and Angiotensin II-receptor expression and the capacity to normalize NO biosynthesis may determine whether ACE-inhibitors, Angiotensin II Receptor antagonists and other substances affect angiogenesis. Reconstitution of a normally vascularized tissue by reversal of impaired angiogenesis with drugs such as ACE inhibitors and AT1 receptor antagonists may contribute to successful treatment of hypertension-associated target organ damage, e.g. left ventricular hypertrophy.

Introduction

Arterial Hypertension is associated with altered function and structure of big and small vessels. Abnormal regulation of vasomotor tone, enhanced vasoconstriction, reduced vasodilation, structural alterations of arteries, microvessels and microvascular networks substantially contribute to hypertension and hypertension-associated target organ damage. Structurally, hypertension increases the thickness of artery walls, increases the wall-to-lumen ratio, and alters the composition of the arteries. In microvessels, hypertension reduces the numbers of small arteries and capillaries [1, 2]. Specifically, primary hypertension of animals and humans is consistently associated with microvascular rarefaction [1, 2]. Hypertension can also cause microvascular rarefaction by two fundamental mechanisms - either destruction of microvessels because of increased blood pressure or, as recently detected, via impaired angiogenesis [2, 3]. The link between hypertension and impaired angiogenesis is the main focus of this review.

The centre of attention in antihypertensive therapy has been to induce vasodilation [3]. In addition, antihypertensive therapy has increasingly been targeted at target organ damage including changes of vascular structure and left ventricular hypothrophy [4]. Attention has recently also been directed at reducing or even reversing microvascular rarefaction [3]. Interestingly, several antihypertensive drugs, which were initially designed to promote vasodilation, are now known to improve altered structure of arteries and microvascular networks. These latter effects require time whereas changes of vascular tone occur quickly.

To reverse microvascular rarefaction by antihypertensive therapy, microvascular networks that have been destroyed in response to high blood pressure or that have not formed because of hypertension-associated impaired angiogenesis need to be established or reestablished.

Several observations suggest that microvascular rarefaction in hypertension may also be due to impaired angiogenesis and that this can be reversed: (i) Microvascular rarefaction precedes manifest elevation of blood pressure and hypertension in persons with a family history of hypertension and in animal models of hypertension [1]; (ii) 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; (iii) Induction of arterial hypertension by NO-biosynthesis inhibitors leads to impaired generation of a vascularized connective

tissue *in vivo*, i.e., impaired angiogenesis; and (iv) Antihypertensive treatment can reverse microvascular rarefaction in animal models of hypertension *in vivo*.

In this review we will discuss some basic mechanisms of angiogenesis and their involvement in hypertension along the four points mentioned above.

Angiogenesis

Formation of new blood vessels involves three fundamentally different processes; vasculogenesis, angiogenesis, and arteriogenesis.

In vasculogenesis, blood vessels form de novo during embryogenesis. Undifferentiated precursor cells (angioblasts) differentiate to endothelial cells which then assemble to primitive vascular networks [5-8].

Angiogenesis refers to the formation of new blood vessels from existing microvessels. It serves the supply of oxygen, nutrients, and the removal of waste. The key sequence of events in angiogenesis can be briefly summarized as follows: Capillary blood vessels consist of endothelial cells and pericytes. These two cell types carry information to form new tubes, branches, and entire new capillary networks. Several stimuli such as hypoxia due to insufficient tissue perfusion induce angiogenesis via release of angiogenic molecules [6-9]. Tissue hypoxia occurs when the vascular supply is overwhelmed by increasing metabolic demands. For example, hypoxia emerges in growing tumors, myocardium in coronary heart disease, striated muscles in peripheral artery disease or in ischemic parts of the retina in diabetic patients.

Tissue hypoxia promotes the release of molecules that induce angiogenesis and a vascularized connective tissue stroma. These molecules include Vascular Endothelial Growth Factor (VEGF), Platelet-derived Growth Factor (PDGF), and Fibroblast Growth Factor (FGF) [10, 11]. Initiation of angiogenesis and the release of angiogenic molecules induces vasodilation, increased vascular permeability, and expression of proteolytic enzymes [8]. Release of Nitric Oxide (NO), for example in response to hypoxia or specific disease states, is important in mediating initial vasodilation in angiogenesis [8, 12]. NO can also upregulate VEGF leading to increased vascular permeability and angiogenesis [13]. In addition to NO and VEGF many other molecules are involved in initiation of angiogenesis (see below) [6, 8, 9].

Next, endothelial cells begin to proliferate and to migrate towards the source of angiogenic molecules. The basement membrane and subendothelial extracellular matrix are subsequently degraded. Then endothelial cells reassemble along tracks of

extracellular matrix [14], form cords and finally tubes after acquiring a lumen [5-8]. Each of these steps is highly regulated by an extensive number of different interacting intracellular and extracellular molecules and cellular receptors [6, 8, 9]. Thus, angiogenesis contributes to expansion and remodeling of the vascular network by proliferation and sprouting of endothelial cells, addition of pericytes, and by splitting of capillaries [5-8]. Finally, capillary vessels coalesce to a complex vascular network.

Angiogenesis is usually turned on for brief periods (days) and then completely inhibited. However, in some processes and diseases the balance of angiogenesis is persistently tipped towards upregulation (cancer, diabetic retinopathy) [7] or downregulation (arterial hypertension: see below) [15].

During subsequent arteriogenesis some vessels reassemble and develop a multilayered muscular coat which provides blood vessels with viscoelastic and vasomotor properties. New arteries form which adapt tissue perfusion to changing needs [5-8]. Arterogenesis is a process that is both phenomenologically and mechanistically totally different from angiogenesis [16-18].

Angiogenesis occurs in a broad array of physiologic and pathologic processes [5-7, 9, 19]. For example, angiogenesis is a prerequisite of normal tissue growth, embryogenesis, wound healing, the ovarial - and menstrual cycle. Furthermore, angiogenesis promotes cancer growth and metastasis, diabetic retinopathy, and instability of atherosclerotic plaques [5-7, 9, 19]. On the other hand, angiogenesis is present but usually insufficient to restore normal left ventricular function in ischemic myocardium due to coronary heart disease or ischemic skeletal muscle due to peripheral artery disease [20-22].

Rarefaction of microvessels in hypertension

Arterial hypertension is characterized by reduced NO biosynthesis, increased vasoconstriction, and by reduced microvascular density, i.e., rarefaction of arterioles and capillaries. Microvascular rarefaction is found in animal models of hypertension and in human hypertensives [1, 2]. Specifically, decreased numbers of small arterioles have been found in spontaneously hypertensive rats [23, 24], and microvascular rarefaction has been associated with high salt intake in skeletal muscle of Dahl salt sensitive rats [25, 26]. Microvascular rarefaction has also been detected in hypertensive patients [1, 27] and even in offspring of hypertensives [1].

Microvascular rarefaction contributes to increased peripheral vascular resistance and in consequence to the development of chronic arterial hypertension [1]. It also impairs target organ blood flow, for example to the myocardium [2]. Indeed, rarefaction of intramyocardial vessels is one of the most relevant structural alterations in hypertension-induced left ventricular dysfunction, hypertrophy, and ischemia [1]. Interestingly, microvascular rarefaction induced by high sodium diet in Sprague-Dawley-reduced-renal-mass rats can be reversed after return to a low sodium diet upon normalisation of blood pressure [28].

Elevated blood pressure is well known to destroy microvessels which contributes to microvascular rarefaction [23, 24]. However, blood pressure-independent mechanisms can contribute to arteriolar rarefaction in arterial hypertension [1, 29]

Interactions between hypertension and angiogenesis

As mentioned above, offspring of patients with hypertension, that have not yet developed arterial hypertension themselves, show rarefaction of microvessels [1]. Deficient angiogenesis, i.e., inadequate upkeep and development of a vascularized connective tissue in target organs, may reduce microvascular density in persons prone to hypertension [1]. Hypothetically, impaired angiogenesis in patients prone to hypertension may evolve because of genetic disposition, deficient placental and embryonal vascular development and thus low birth weight, and also impaired postembryonic vascular growth in general and in target organs (for example hypertrophying myocardial tissue). Inadequate formation of new blood vessels, i.e., impaired angiogenesis, may thus be intrinsically associated with hypertension and

development of hypertension-dependent target organ damage [1-3, 7, 9]. However, this has not been elucidated yet in detail.

Mechanistically, interactions of angiogenesis and hypertension may involve amongst others blood pressure itself, nitric oxide, angiogenic molecules, and the Renin-Angiotensin-Aldosteron-System (RAAS). In an own series of experiments we found that blood pressure itself did not seem to affect the extent of angiogenesis in Dahl salt sensitive rats "Fig. (**1**)" [15]. However, decreased NO biosynthesis reduced angiogenesis in the same study in a rat model of secondary hypertension "Fig. (**1**)" [15]. Thus, rather than from elevated blood pressure itself, impaired angiogenesis in hypertension may result from metabolic changes associated with hypertension such as impaired NO biosynthesis [15], activation of the RAAS, and other factors [1, 15].

Recently, increased plasma levels of VEGF have been found in hypertensive patients [30, 31]. Specifically, VEGF and its soluble receptor sFlt-1 in patients with essential hypertension revealed an elevation of VEGF [30, 31] and a reduction of sFlt-1 levels [30] compared to normotensive control patients. Treatment of hypertension reduced plasma levels of VEGF [30, 31] and increased plasma levels of sFlt-1 [30]. Therefore, antihypertensive therapy seems to normalize deregulated angiogenic markers and possibly restore the normal potential for angiogenesis.

Interactions of hypertension and angiogenesis may also be present in diabetes. Diabetes is closely linked to some forms of essential hypertension [32, 33]. Hypertension contributes to diabetic microvascular and macrovascular complications [33-36]. These complications are often related to aberrant angiogenesis. Excessive angiogenesis in response to retinal microvascular damage and ischemia plays a role in diabetic retinopathy, and impaired angiogenesis contributes to diabetic neuropathy, poor wound healing in diabetic patients, embryonic vasculopathy in pregnancies complicated by maternal diabetes [37]. VEGF and sFlt-1 levels of diabetic patients in plasma are modified may be associated with deregulated angiogenesis [38, 39].

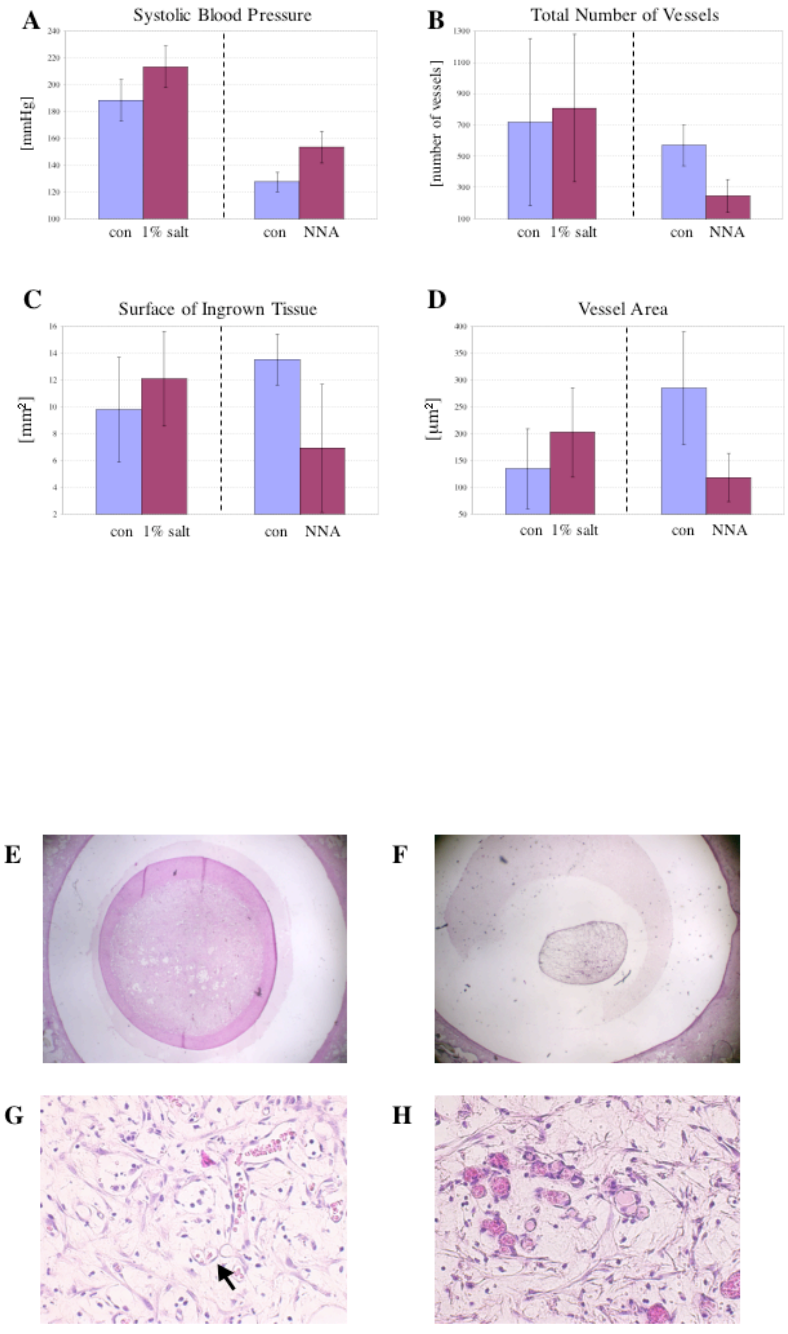

Figure 1: Inhibition of NO biosynthesis but not elevated blood pressure reduces angiogenesis [15].

Angiogenesis *in vivo* was investigated in two models of secondary arterial hypertension using a wound chamber model *in vivo* [15, 60, 70]. Arterial hypertension was induced by salt feeding of DSS (dahl salt sensitive) rats and inhibiting Nitric Oxide biosynthesis by Nw-Nitro-L-Arginine (NNA) in WKY rats [15].

Panels A - D show blood pressure (A) and morphometric parameters (C-D). Blood pressure increases significantly in response to salt-feeding and Nitric Oxide biosynthesis inhibition (con = control rats, 1% salt = salt-feeding with 1% salt in the drinking water, NNA = 150 mg Nw-Nitro-L-Arginine/ l H_2O in the drinking water). The total number of vessels (B), the surface of ingrown tissue (C), and the vessel area (D) decrease by inhibition of NO biosynthesis but not by high blood pressure in response to salt feeding [15].

Panel E to H show sections through wound chambers. Panel E shows a cross section of a wound chamber of a control rat with tissue ingrown into the wound chamber at 20x magnification. Similar patterns are also observed for salt-fed rats (not shown). Inhibition of NO biosynthesis massively reduces the surface of ingrown tissue (F). Panel G shows a vascularized wound chamber at higher magnification (400x), as observed in control groups and salt-fed rats. Inhibition of NO biosynthesis (Panel H) reduces the total number of blood vessels and decreases their size [15]. Arrows in G and H point to blood vessels.

Angiogenesis and Nitric Oxide (NO)

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* [40, 41]. 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 [42-44]. As mentioned above, vasodilation precedes sprout-formation at initiation of angiogenesis [8, 12]. This vasodilation may be NO-mediated, and NO-mediated vasodilation may be a prerequisite of elongation, migration, and proliferation of endothelial cells, cellular actions which are all required for angiogenesis (see above) [12].

NO is also involved in regulation of angiogenesis by influencing the activities of different angiogenic growth factors. Most evidence for the involvement of NO in angiogenesis has been generated with VEGF, an angiogenic growth factor [6, 7, 9, 45]. VEGF induces NOS and NO biosynthesis which are required for subsequent angiogenesis [46-50]. Conversely, NO also activates VEGF mRNA and protein expression [13, 51]. The importance of NO in angiogenesis has also been demonstrated in knockout mice. Both inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) knockout mice display impaired angiogenesis [52-55]. Recently, iNOS has been found to drive angiogenesis dependent skin flap survival; in a mouse model in which survival of a skin flap entirely depends on angiogenesis to

provide an arterial blood supply, targeted disruption or selective inhibition of the iNOS gene reduces skin flap survival [53]. In a rat model of ischemic cardiomyopathy needle-induced transmyocardial revascularization (TMR) iNOS may contribute to the angiogenic response of TMR [56]. In this model a mechanical injury using needle puncture leads to increased expression of myocardial iNOS and is associated with increased vascular density [56]. Furthermore, ACE inhibition and Bradykinin induce eNOS in ischemic hind limbs of mice [57], in rat endothelial cells [58], and in rabbit coronary postcapillary venular endothelial cells [59] and thereby elicit angiogenesis [57-59]. Beside VEGF, other angiogenic growth factors can also require NO. FGF for example requires NO in mesenterial angiogenesis *in vivo*; inhibition of NO formation in an own study diminishes unstimulated and bFGF-stimulated angiogenesis in the mesenterium of rats [60].

NO is also necessary for wound healing, of which angiogenesis is an integral part [61-63]. During early stages of wound healing end products of NO synthesis (nitrite, nitrate) are elevated [64]. Furthermore, nitric oxide synthase (NOS) inhibitors delay wound healing [65, 66]. Both inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) have directly been implicated in wound healing and are required for proper endothelial cell migration, proliferation, and differentiation [52, 54]. Tumor tissues express elevated levels of total NOS, iNOS and cGMP [12, 67, 68], and inhibition of NO biosynthesis by N^{w}-nitro-L-arginine-methyl ester (L-NAME) reduces tumor-induced angiogenesis [12, 67-69].

The essential role of NO for angiogenesis is also supported by data in many other diseases. However, we next focus on diseases with a relevant hemodynemic aspect. Portal hypertension is defined as an increased pressure in the portal vein and its tributaries. However, in the systemic circulation portal hypertension has many characteristics that are quite the opposite of arterial hypertension. Portal hypertension leads to elevated NO biosynthesis, systemic vasodilation, and a decrease in blood pressure. Interestingly, we found portal hypertension to increase NO dependent angiogenesis in the mesenteric vascular bed of rats [70]. In other words, portal hypertension leads to increased angiogenesis via NO biosynthesis [70].

In a further series of experiments we found that decreased NO biosynthesis reduced angiogenesis in a rat model of secondary hypertension “Fig. (**1**)” [15]. Interestingly, blood pressure itself did not affect the extent of angiogenesis “Fig. (**1**)” [15].

Therefore, arterial hypertension may be associated with reduced angiogenesis due to impaired NO biosynthesis [15].

All of these results support a central role for NO in angiogenesis and also arterial hypertension-associated impairment of angiogenesis. However, the specific cellular signaling mechanisms by which NO affects angiogenesis are not yet well characterized. Especially, it is still unclear whether NO acts directly on endothelial cells or via growth factor dependent effects [71, 72]

Angiogenesis and the Renin-Angiotensin-Aldestoron System

The Renin-Angiotensin-Aldosteron System (RAAS) plays a central role in blood pressure control. It contributes to the pathophysiology of hypertension and to target organ damage [73-75]. Target organ damage includes vascular remodeling, glomerular damage to the kidney, and remodeling and hypertrophy of the left ventricle (left ventricular hypertrophy) [73-75]. The RAAS also affects angiogenesis "Fig. (**2**)". A main effector molecule of the RAAS, Angiotensin II (Ang II), appears to act as an angiogenic factor [76]. Thus, angiogenic effects of Ang II have been shown in different models of angiogensis such as the chick chorioallentoic membrane [77], sponge implants in rodents [78] and the rat cornea model [76]. Ang II induces targeted migration of endothelial cells [79] and pericytes [80]. Endothelial cells express high levels of both Ang II receptor subtypes, AT1 and AT2 [81]. Hence, Ang II-induced migration via Ang II receptors appears to be a critical element for its angiogenic effect. However, the exact mechanisms by which Ang II induces angiogenesis are not fully elucidated yet.

The AT1 receptor mediates growth promoting effects of Ang II in endothelial cells, while activation of the AT2 receptor leads to inhibition of endothelial cell proliferation [81-85]. However, the AT2 receptor might also be important for mediating angiogenesis [86] "Fig. (**2**)". On the basis of the currently available studies it is not yet possible to fully understand effects of angiogenesis mediated via the AT2 receptor [81-85]. This may be due to distinct model systems used to investigate the role of the AT2 receptor [81-85]. There are other possible explanations for the potentially conflicting effects mediated via the AT2 receptor, i.e., inhibition of endothelial proliferation on the one hand [81-85] and induction of angiogenesis on the other hand [86]; AT1 and AT2 receptors are sequentially expressed during

microvascular maturation and therefore effects of endogenous Ang II on angiogenesis may depend on the balance of their local expression [76, 87].

The RAAS is also involved in tumor angiogenesis. For example, renin can be produced by both benign and malignant tumors [88-93]. In some of these tumors, increased presence of renin is associated with the degree of vascularization [76]. Also, some tumors express Ang II receptors [88, 93]. Thus, Ang II may hypothetically act as a paracrine-autocrine factor that promotes tumor growth [76]. Inhibition of Ang II should therefore theoretically repress tumor growth via repression of angiogenesis. Indeed, different ACE inhibitors reduce angiogenesis in chemically-induced or implanted tumors in rat - and mouse models [76, 79, 94, 95], partially due to inhibition of proteases other than ACE [76, 79, 94, 95]. Interestingly, first clinical observations have also suggested reduced rates of cancer in patients treated with ACE inhibitors [96, 97].

At the same time and in contrast to tumors, ACE inhibitors may have angiogenic effects in normal tissue [98, 99]. Angiogenic effects of ACE inhibition result probably from Bradykinin (BK). ACE catalyzes the conversion of Ang I to Ang II and the breakdown of BK to kinin degradation products. Therefore, pharmacological effects of ACE inhibition on angiogenesis may in part be mediated via inhibition of Ang II or also via accumulation of BK and subsequent BK-induced NO biosynthesis. Indeed, several lines of evidence support a role of BK in modulating angiogenesis. BK activates angiogenesis via FGF in endothelium from bovine coronary postcapillary venules [100] and enhances angiogenesis in rat subcutaneos-sponge granuloma in synergism with interleukin-1 [101].

BK acts via its two receptors, the BK1 and BK2 receptors. A substantial body of evidence suggests that BK signal transduction for endothelium-dependent vasodilation is mediated via the BK2 receptor [102]. The BK2 receptor is usually expressed constitutively in various tissues [103]. However, myocardial ischemia can trigger up-regulation of BK2 receptors in male Sprague Dawley rat hearts. [104]. The angiogenic effect of ACE inhibition appears to be mainly mediated via BK and the BK2 receptor [57, 58, 105]. Stimulation of the BK2 receptor induces activation of eNOS and secretion of the angiogenic factor VEGF [57, 58, 105]. In BK2 knock out mice, the capability of ACE inhibition to activate eNOS and induce angiogenesis is abrogated [57]. This supports the concept that angiogenic effects of ACE inhibition

are mediated via BK2-induced eNOS activation [57, 58, 105]. However, in addition to the BK2 receptor, the BK1 receptor may also mediate angiogenesis. The BK1 receptor is undetectable under physiological conditions and strongly up-regulated following tissue injury or inflammation [103]. For example, the BK1 receptor is upregulated in ischemic skeletal muscle of mice [106] or ischemic myocardium of rats [107]. In line with this observation, abrogation of BK1 receptor signaling inhibits an angiogenic response in a murine model of hindlimb ischemia [108]. Conversely, delivery of BK1 receptor agonist enhances collateral vascular growth in ischemic skeletal muscle of mice [108]. *In vitro*, BK1 receptor activation stimulates endothelial cell proliferation and survival [108]. Furthermore, BK acting via the BK1 receptor up-regulates the angiogenic factor FGF-2 via the NOS pathway [59, 100]. Taken together, BK seems to be a powerful angiogenic stimulus *in vivo* and *in vitro* "Fig. (**2**)".

Recent data also suggest a role for endogenous BK in tumor angiogenesis and tumor growth [109, 110]. The angiogenic properties of endogenous BK in tumor growth seem to be mediated by the BK2 receptor [109, 110]. In mice bearing sarcoma 180 cells daily administration of BK2 receptor antagonists suppresses the increment in angiogenesis and tumor weight, whereas BK1 receptor antagonists do not affect tumor growth [109, 110]. In these mice BK promotes angiogenesis by increasing vascular permeability and by promoting up-regulation of VEGF [110].

Evidently, the data on ACE-inhibition and angiogenesis are not congruent. It is still unclear why ACE inhibition might affect angiogenesis in divergent ways; in tumor angiogenesis ACE inhibition appears to inhibit angiogenesis and to perhaps repress cancer [76, 79, 94-97]. On the other hand ACE inhibition seems to lead to strong angiogenic effects mediated via BK [57-59, 100, 105] and other effector molecules such as FGF-2 [59, 100], eNOS [57, 58], VEGF receptors [58, 105], PKC [105] in different cell types and in different models. These discrepancies may be due to the presence or absence of different elements of the RAAS system in tumor tissue versus non tumor tissue. Hypothetically, ACE-inhibition and subsequent reduction of Ang II therefore overrides angiogenic effects of scarce BK accumulation in tumors. In non tumor tissues Renin and Ang II may hypothetically be less involved in regulating angiogenesis. Thus, accumulation of BK in response to ACE inhibition may be in the foreground in these situations and result in angiogenesis "Fig. (**2**)".

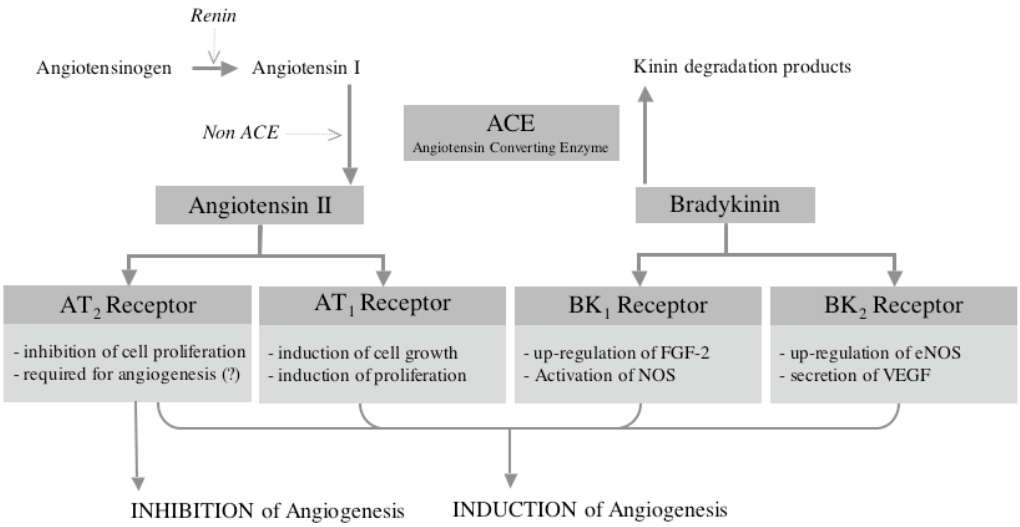

Figure 2: Schematic representation of the Renin-Angiotensin-Aldosteron-System and pathways that can affect angiogenesis.

Treatment of hypertension and improvement of angiogenesis (Table 1)

Antihypertensive therapy reduces cardiovascular events such as stroke and coronary heart disease [111]. Main goals of the therapy are to improve morbidity, quality of life, and mortality by inducing vasodilation, reducing vasoconstriction, and reverting target organ damage. Some target organ damage caused by hypertension is related to the microcirculation [111]. Therefore, antihypertensive therapy should also target the microcirculation with, amongst others, the goal to reverse microvascular rarefaction. Amongst the widely used classes of antihypertensive agents, ACE inhibitors and potentially AT1 receptor blockers are the most promising to revert microvascular rarefaction.

As extensively discussed in the previous section, ACE inhibition leads to accumulation of BK which has a strong angiogenic potential mediated via both its receptors. Accumulation of BK leads to endothelium-dependent vasodilation [102] and a lowering of blood pressure. The beneficial effect of ACE inhibition on the microvasculature is probably due to angiogenesis via BK and other molecules such as FGF, VEGF, eNOS and PKC (see section above). In the following we illustrate the

situation with a few examples of more clinically-oriented *in vivo* models; the ACE inhibitor perindopril increases vessel density and capillary number in ischemic hindlimbs of mice [57]. The BK2 receptor accompanied by an increase in eNOS protein level mediates this increase of vessel density [57, 58]. Spirapril, another ACE inhibtor, substantially increases myocardial capillary microvascular density in spontaneously hypertensive rats [98, 99]. Spirapril also improves left ventricular function by reducing its thickness and its hypertrophic weight [98, 99]. It is not known whether improved function of the left ventricle is also due to enhanced angiogenesis. Another ACE inhibitor, quinaprilat, promotes angiogenesis in a rabbit model of hindlimb ischemia *in vivo* [98].

Much is known about Angiotensin II and its possible involvement in angiogenesis (see section above). However, only little data is available on Angiotensin II receptor blockers, which are commonly used to treat hypertension. Angiotensin II decreases capillary density of the left ventricle in the long term [112]. AT1 receptor blockage with candesartan in stroke prone spontaneously hypertensive rats increases left ventricular capillary density and improves function of the left ventricle after the animals were treated with Angiotensin II to induce cardiac hypertrophy [113]. Similarly, losartan prevents a decrease in the capillary density in the microvasculature of the left ventricle of rats treated with Angiotensin II [112]. However, AT1 receptor blockage with Losartan does not improve capillary density in stroke-prone spontaneously hypertensive rats that were not treated with Angiotensin II [114]. These data suggest that AT1 receptor blockage is only able to revert capillary density to normal if Angiotensin II was increased in the model [114].

Even less is known on other classical antihypertensive agents such as calcium antagonists, □- and □-blockers, and diuretics. But recent data suggest that these agents may also influence angiogenesis. The calcium antagonist verapamil induces angiogenesis in the myocardium of rats with nitric oxide blockage [115]. Treatment with several calcium antagonists (verapamil, nifedipine, and nimodipine) increases vascular density on the chick chorioallantoic membrane [116]. In spontaneously hypertensive rats, nifedipine expands the coronary capillary network [117]. Benidipine, a lon-acting calcium channel blocker, improves capillary density in the left ventricle of hypertensive rats [118]. However, benidipine also suppresses expression of angiogenic growth factors in a diabetic rat model [119]. In summary,

calcium antagonists may reduce mircrovascular rarefaction, i.e., possibly improve angiogenesis. However, this has not been extensively studied yet.

Virtually nothing is known about the effects of β- and α-blockers on angiogenesis, although β-blockers are widely used antihypertensive agents. α_1-Adrenoreceptor blockers such as prazosin are known to increase capillary density in rat skeletal muscle [120, 121]. Currently, third-generation β-blockers such as Nebivolol and Carvedilol are more widely investigatd. These β-blockers have additional vasodilating effects. Nebivolol for example, is a highly selective β_1-adrenergic receptor antagonist with additional β_1-adrenergic independent vasodilating properties mediated by increased NO biosyntheses [122]. It is unclear whether these regents can affect angiogenesis.

Effects of diuretics on angiogenesis have barely been reported. Spironolactone, for example, inhibits proliferation of smooth muscle cells [123] and also proliferation of endothelial cells [124] *in vitro*. However, no effect of spironolactone is observed on migration and rearrangement of endothelial cells into capillary-like structures in a matrigel model *in vitro* [124].

First studies have examined the combination of different antihypertensive agents and their effects on angiogenesis. Promising results were obtained by combination of ACE inhibitors with diuretics [125, 126]. In stroke prone spontaneously hypertensive rats a combination of a low dose of the ACE inhibitor perindopril and low doses of the diuretic indapamide increases capillary density in the cardiac ventricles [125]. The combination of both agents is more effective than administration of each agent alone [125]. The same combination of perindopril and indapamide increases neovascularization in ischemic hindlimbs of rats [126]. These results suggest that combinations of different antihypertensive agents may be a promising possibility to improve target organ damage because of hypertension and microvascular rarefaction.

Table 1. Effects of antihypertensive drugs on angiogenesis

Drug	Species	Organ and Model	Effect on vascularisation or angiogenesis	Reference
ACE inhibitors				
Perindopril	Mouse	Ischemic hind limb	Increases vessel density.	Silvestre et al. Circ Res 2001
Spirapril	Rabbit	Ischemic hind limb	Increases vessel density of capillaries.	Fabre et al. Circulation 1999
	SHR	Heart – LV	Increases vessel density of myocardial capillaries.	Oliveiti et al. J Cardiovasc Pharmacol 1993
Quinaprilat	Rabbit	Ischemic hind limb	Increases vessel density of capillaries.	Fabre et al. Circulation 1999
AT 1 receptor blockers				
Candesartan	SHRSP	Heart – LV	Increases vessel density of capillaries in Ang II pretreated rats.	Xie et al. Clin Exp Hypertens 1999
Losartan	Rat	Heart – LV	Prevents decrease of vessel density of capillaries in Ang II pretreated rats.	Sabri et al. Hypertension 1998
Losartan	Rat	Heart – LV	No change of vessel density of capillaries in rats not pretreated with Ang II.	Gohlke et al. Hypertension 1997
Calcium antagonists				
Verapamil	Rat	Myocardium	Increases vessel density of capillaries in L-NAME pretreated rats.	Meirelles Pereira et al. Pathol Res Pract 2000
	Chick	Chorioallantoic membrane	Increases vessel density.	Dusseau et al. Int J Microcirc Clin Exp 1993
Nifedipine	Chick	Chorioallantoic Membrane	Increases vessel density.	Dusseau et al. Int J Microcirc Clin Exp 1993
	SHR	Heart	Increases vessel density of capillaries.	Rakusan et al. Hypertension 1994
Nimodipine	Chick	Chorioallantoic membrane	Increases vessel density.	Dusseau et al. Int J Microcirc Clin Exp 1993
Benidipine	Hypertensic rat	LV	Increases vessel density of capillaries.	Kobayashi et al. Am J Hypertension 1999
	Diabetic rat	Heart	Suppression of angiogenic growth factors VEGF, bFGF and TGF-β1.	Jesmin et al. Diabetologia 2002
α- and β-blockers				
Prazosin	Rat	Ischemic skeletal muscle	Increases vessel density of capillaries.	Pulgenzi et al. Eur j Vasc Endovasc Surg 1998
Diuretics				
Spironolactone	in vitro	HUVEC	Inhibits proliferation of endothelial cells.	Guggino et al. Arch Mal Coeur Vaiss 2002
Comination of antihypertensive agents				
Perindopril &	SHR	Heart	Increases vessel density of capillaries.	Rakusan et al. Microvasc Res 2000
Indapamide	Rat	Ischemic hind limb	Increase of neovascularization.	Silvestre et al. J Pharmacol Exp Ther 2002

SHR: spontaneous hypertensive rats
SHRSP: stroke prone spontaneously hypertensive rats
LV: left ventricle
L-NAME: Nitric oxide inhibitor (NG-Nitro-L-arginine-methyl ester)
HUVEC: human umbilical vein endothelial cells

Table 1: Effects of Antihypertensive Drugs on Angiogenesis

Conclusion

There is little doubt about the evidence for abnormal angiogenesis in cardiovascular disease, and increasing evidence points towards some role in the pathogenesis of hypertension. Many of the currently available antihypertensive agents may have beneficial effects on angiogenesis and thus potentially improve microvascular rarefaction, corresponding target organ damage and function.

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A versatile *in vitro* assay for investigating angiogenesis of the heart

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Abstract

Neovascularization in the heart is usually investigated with models of angiogenesis *in vivo*. Here we present a simple model that allows investigating heart angiogenesis in mice and rats *in vitro*.

Small pieces of left ventricular myocardium were cultured in three-dimensional fibrin gels for 10 days. A single mouse heart allowed assessing 24 conditions, each tested in octuplicates. Rat recombinant $VEGF_{164}$, human recombinant bFGF and human recombinant PDGF-BB were used under normoxia (21% O_2) and hypoxia (3% O_2) and outgrowth of endothelial sprouts from heart pieces was quantified. In 4-week-old OF1 mice, endothelial sprouts formed spontaneously. In contrast, in 12-week-old adult mice, virtually no sprouts formed under normoxia. Under hypoxia, sprout formation increased substantially. Different growth factors induced formation of distinct patterns of sprouts and unorganized single cells. Sprouts were composed of endothelial cells with smooth muscle cells or pericytes interacting with them, as assessed by inmunohistochemistry.

Taken together, our model is suited for investigation of angiogenesis of the heart *in vitro.* It may allow performing extensive series of experiments *in vitro* including rapid screening of pharmacological compounds and assessment of mechanisms of heart angiogenesis in transgenic animals in an easy straightforward manner.

Introduction

Ischemia of the left myocardial ventricle results from coronary heart disease or left ventricular remodelling and microvascular rarefaction in hypertension. Angiogenesis, serves to reduce tissue ischemia, i.e., hypoxia. Unfortunately, intrinsic angiogenesis rarely fully compensates for the blood supply lost by occlusion of coronary arteries [1]. A number of growth factors induce angiogenesis *in vivo* and *in vitro* and stimulate endothelial migration or proliferation *in vitro* [2, 3]. Angiogenic growth factors, such as $VEGF_{164}$ (Vascular Endothelial Growth Factor) and bFGF (basic Fibroblast Growth Factor) are released in response to hypoxia [4] and induce angiogenesis in the heart [5]. Furthermore, expression of $VEGF_{164}$ receptors flt-1 and flk-1, and FGF receptor FGFR-1 increases in both acute [6] and chronic [7] myocardial ischemia. Similarly, Platelet-derived Growth Factor (PDGF) limits the extent of myocardial infarction via angiogenesis [8]. However, not much is known about the mechanisms of PDGF-BB-mediated angiogenesis in the heart [8, 9].

Therapeutic angiogenesis aims at using angiogenic growth factors and other therapeutic modalities to further enhance or promote new or collateral blood vessel formation in order to reduce myocardial ischemia and thus improve myocardial function [5, 10]. Recent first clinical trials with administration of different angiogenic growth factors to the human heart have not been very successful [11, 12]. For this reason, angiogenesis in the heart and possibilities to affect it need to be further investigated.

Effects of angiogenic molecules on myocardial angiogenesis have been characterized in a number of animal models *in vivo* [13-15]. These studies have demonstrated enhanced collateralization in ischemic tissues, improved coronary blood flow, improved functional capacity, and reduction in infarct size in response to growth factors. Currently, most experiments are performed *in vivo* since no appropriate *in vitro* model is available. *In vivo* experiments require large number of animals, are difficult to perform and are often associated with pain to the animals. 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 needed. Thus, the aim of this study

was to investigate and validate a model of angiogenesis of the heart *in vitro* and to assess the response of heart angiogenesis to known important angiogenic stimuli.

Results

Basic conditions of the heart *in vitro* assay

First we established standard conditions for the *in vitro* heart angiogenesis assay using small (0.5 to 1 mm^3) pieces of left myocardial tissue of mouse or rat hearts embedded in a fibrin gel. We investigated two cell culture media (Dulbecco's minimal essential medium, DMEM, and MCDB 131) (data not shown) with different concentrations of foetal calf serum (FCS; 1, 2.5, 5 and 10% FCS; Fig. 1A) and evaluated the required incubation period (Fig. 1B). DMEM proved to be the optimal medium (data not shown) when supplemented with 5% FCS for our assay in both mouse and rat hearts. This concentration is the maximal FCS concentration that shows no significant growth in comparison to diluent and the one that shows the largest ratio between stimulated and un-stimulated responses under hypoxic conditions.

Under hypoxia sprouts started to form 3 days after incubation and increased in number up to day 10 of incubation (Fig. 1B). Virtually no *in vitro* angiogenesis was observed under normoxic cell culture conditions (21% O_2) (Fig. 1A and 4).

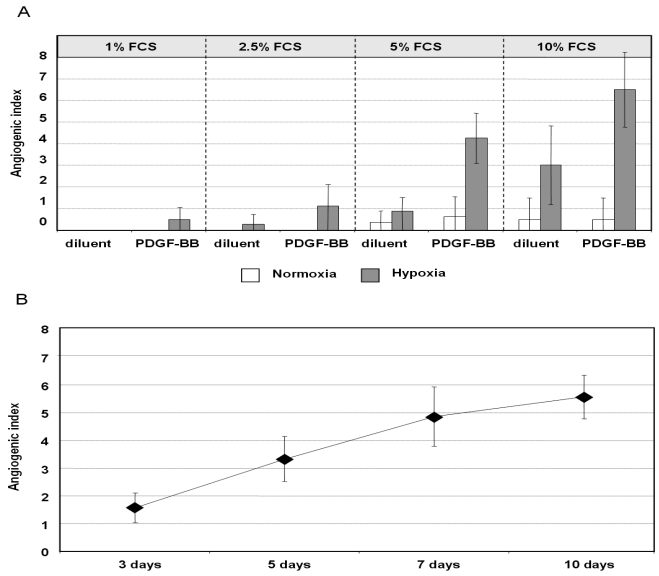

Fig. 1. Determination of standard conditions for heart angiogenesis *in vitro* in adult mouse hearts.

(A) The serum concentration of the medium (DMEM) that covers the gel influences the degree of sprout formation in response to human recombinant hrPDGF-BB (10 ng/ml) under normoxia (21% O_2) and hypoxia (3% O_2). Sprouts were quantified with a standardized scale ranging form 0 to 8 (angiogenic index). Medium containing 5% Fetal Calf Serum (FCS) was found to be optimal because control sprouting (diluent) is low and sprouting can be amplified by addition of hrPDGF-BB. (B) Spouting after 3, 5, 7 and 10 days in culture with addition of 5% FCS and 10 ng/ml hrPDGF-BB under hypoxia. Sprouting increases continuously up to 10 days of incubation. Data points represent the mean of a representative experiment ± SD (A and B).

Angiogenesis of the heart *in vitro* in comparison to a well-established assay of aortic angiogenesis

To further characterize the assay of heart angiogenesis *in vitro* we compared it with a well-established assay of angiogenesis *in vitro* that uses rat *aortae* [9, 16].

Angiogenesis *in vitro* in the heart was more restricted than in the aorta, indicating an organ specific potential for angiogenesis. Under serum free conditions sprouts formed from the rat aorta both under hypoxic or normoxic conditions, but the growth seen under normoxia is negligible (Fig. 2) [9]. In contrast, there was no sprout formation from adult rat hearts under normoxia or hypoxia in serum free culture (Fig. 2). With serum-containing medium, both aorta and heart displayed similar degrees of cellular outgrowth (Fig. 2).

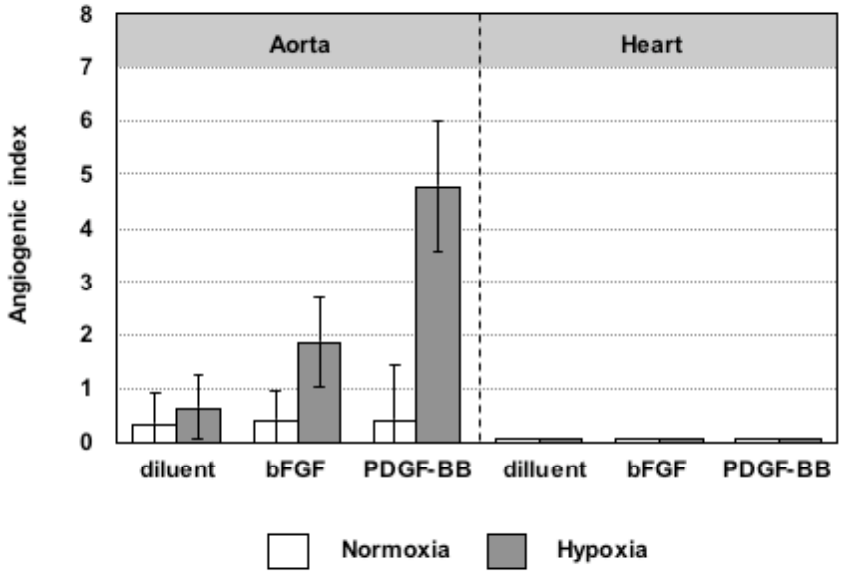

Fig. 2. Angiogenesis *in vitro* of adult rat aorta versus adult rat heart is distinct in a serum free culture.
In order to characterize the response in different organs, we compared growth in heart and aorta of adult rats. This figure shows angiogenesis *in vitro* from pieces of rat aorta and heart after addition of hrbFGF (10 ng/ml) or hrPDGF-BB (10 ng/ml) under normoxia (21% oxygen) or hypoxia (3% oxygen) after 10 days of incubation in a fibrin gel without addition of serum. On the right part sprout formation in rat hearts cultured in 5% FCS is shown as a comparison. A standardized scale ranging from 0 to 8 indicates the degree of cellular outgrowth (angiogenic index). No sprouting is observed under normoxia or hypoxia from rat pieces of heart, but rat aorta sprouted under both conditions. Thus, angiogenesis *in vitro* in aortae does not require the presence of serum in contrast to hearts. Data points represent the mean of a representative experiment ± SD after 10 days of incubation.

Age and angiogenesis

In an additional series of experiments we determined optimal age of animal hearts to be used. We observed that the potential of the heart to form sprouts *in vitro* decreases with the age of the animals (Fig. 3). Under hypoxia, hearts from 4 week old mice (adolescent) displayed earlier and less restricted sprouting when compared to hearts obtained from mice 12 weeks or older (adult). In adult mouse hearts, presence of FCS in the medium was essential for sprout formation whereas adolescent mouse hearts occasionally displayed weak growth even under serum free culture conditions (data not shown). After 7 to 8 days of culture in a fibrin gel, sprout formation from adolescent mouse hearts was already maximal. This degree of sprout formation was only reached after 10 days in hearts of adult mice. As mentioned above, sprout formation was much weaker under normoxia than under hypoxic conditions for both ages tested. Thus, young mice exhibit a significant amount of growth even when un-stimulated and achieve the levels of growth that older mice only reach when stimulated. We conclude that the range of values is much smaller in younger than in the older mice (12 weeks), making the latter the preferable choice for most questions.

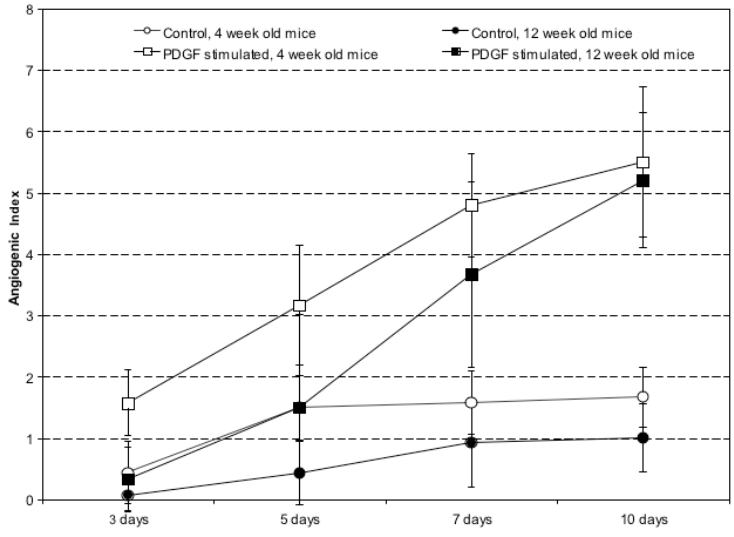

Fig. 3. Sprouting in adolescent mice is less restricted than in adult mice.
Sprouting of heart pieces from adolescent (empty circles and squares) versus adult mice (filled circles and squares) after 3, 5, 7 or 10 days of incubation in hypoxia under standard culture conditions (DMEM 5% FCS) (circles) or after addition of hrPDGF-BB (10 ng/ml) (squares). A standardized scale ranging from 0 to 8 indicates the degree of sprouting (angiogenic index). Angiogenesis of the heart *in vitro* in adolescent mice occurs faster. Adolescent mice display stronger sprouting at all measured time points ($p < 0.05$ Mann-Whitney Test). Also, after addition of hrPDGF-BB growth is stronger and more extensive. At day 10 adult mice hearts achieve similar levels of growth to those seen in adolescent mice. Data points represent the mean of a representative experiment ± SD.

Differential response to angiogenic stimuli

Hypoxia per se induced angiogenesis at a reduced level; all growth factors further enhanced this response (Fig. 4). Specifically, human recombinant Platelet-derived Growth Factor (PDGF-BB) strongly induced sprouting from mouse heart pieces placed under hypoxia (Fig. 4). Compared to hypoxic diluent controls PDGF-BB induced a 5.5 fold increase of sprouting. Under normoxic conditions PDGF-BB induced sprout formation only weakly. In addition to PDGF-BB we also investigated human recombinant basic Fibroblast Growth Factor (bFGF) and rat recombinant Vascular Endothelial Growth Factor 164 ($VEGF_{164}$) (Fig. 4). Compared to hypoxic diluent control bFGF increased sprout formation 1.8 fold and $VEGF_{164}$ 2.3 fold. Under normoxia neither bFGF nor $VEGF_{164}$ induced sprout formation compared to normoxic diluent control (Fig. 4).

Since repression of angiogenesis is of major scientific and clinical interest we also used rapamycin as [9] an inhibitor of angiogenesis in our *in vitro* assay. PDGF-induced sprout formation of hearts *in vitro* was repressed by application of rapamycin, a specific inhibitor of mammalian target of rapamycin (mTOR) signalling. This suggests that mTor may play a role in PDGF-induced angiogenesis of the heart. (Fig. 4).

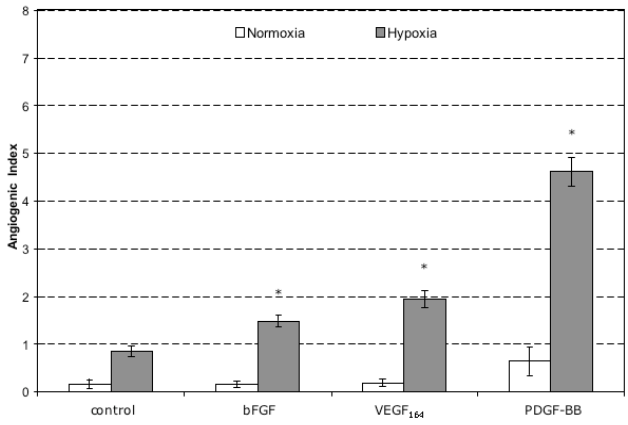

Fig. 4. Angiogenic growth factors induce reproducible sprouting in adult mice hearts after 10 days of incubation.

Growth factors added to the culture medium containing 5% FCS under normoxia (21% O_2) or hypoxia (3% O_2): hrPDGF-BB (10 ng/ml), hrbFGF (10 ng/ml), rr$VEGF_{164}$ (5 ng/ml). Hypoxia significantly increased sprouting in all experimental conditions in comparison to normoxia. Sprouting was maximal in response to hrPDGF-BB under hypoxia (* = $p < 0.05$ Mann-Whitney Test) but significant for all growth factors tested. A low dose (10 nM) of rapamycin

effectively inhibits PDGF-BB-induced sprout formation. A standardized scale ranging from 0 to 8 indicates the degree of sprouting (angiogenic index). Data points represent the mean of five independent experiments ± SEM.

Characterization of morphology

We then characterized outgrowing cells under hypoxic culture conditions in the presence of 5% FCS by analyzing morphology and cell type. To typify single and organized cells, we directly applied fluorescence labelled cell markers on the fibrin gel cultures (Fig. 5). We used an Alexa Fluor labelled isolectin (GSL I –IB_4) as an endothelial cell marker, and Cy3 labelled anti □ smooth muscle actin (□SMA) as a marker for smooth muscle cells and pericytes. In pieces of heart cultured without addition of growth factors some single cells migrated from the heart into the gel. Occasionally, small branches formed spontaneously. A similar phenotype was observed in cultures treated with bFGF (Fig. 5B and E). $VEGF_{164}$ mainly induced outgrowth of un-branched lengthy sprouts (Fig. 5C and F). In contrast, PDGF-BB induced a mixture of all the observed phenotypes (Fig. 5A, D and G-I); a substantial fraction of cells did not organize into sprouts. Still, many cells formed sprouts that were more branched than those observed with $VEGF_{164}$.

All tested growth factors induced organized sprouts of endothelial origin and outgrowth of single cells of mixed origin as detected by cell specific stainings. Most sprouts consisted of cells well organized into longitudinal tracts and branches suggesting cord or tube formation. PDGF-BB induced outgrowth of the largest number of endothelial cells (Fig. 5A). The fraction of non-endothelial cells was proportional to the extent of total outgrowth, independent of the growth factor applied. Some of the □SMA-expressing cells aligned along endothelial sprouts, suggesting early stages of vessel maturation and capillary-like sprout formation (Fig. 5D-I).

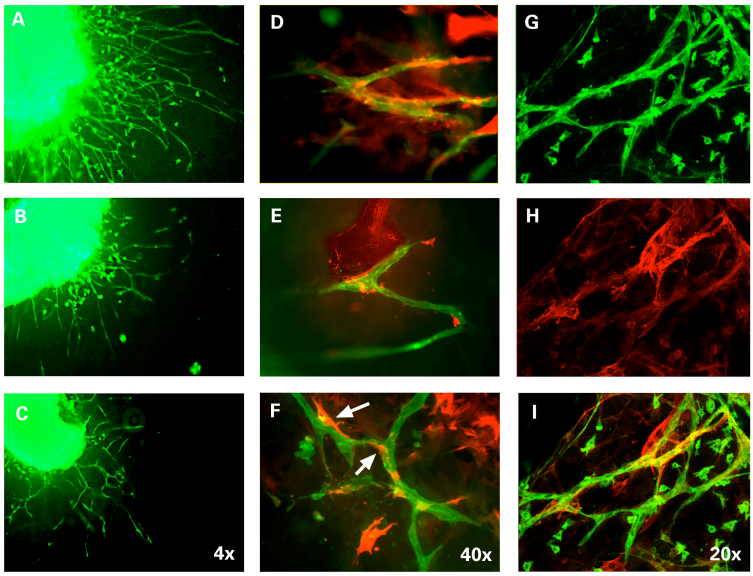

Fig. 5. Different growth factors induce sprouts with distinct morphologies under hypoxia.
10 ng/ml hrPDGF-BB (A), 10 ng/ml hrbFGF (B) and 5 ng/ml rrVEGF$_{164}$ (C) induce substantial endothelial sprout formation as visualized by Fluorescein conjugated GSL I – IB$_4$ (green). All growth factors induce growth of smooth muscle cells or pericytes as visualized by Cy3 conjugated □SMA (red) (D, H and I: hrPDGF-BB, E: hrbFGF and F: rrVEGF$_{164}$). Some smooth muscle cells or pericytes align themselves along endothelial sprouts (D, E, I and arrows in F) suggesting vessel maturation.
We were able to isolate rat heart -but not mouse heart endothelial cells from the gels.

Subcultivation of outgrowing sprouts from rat hearts resulted in enriched endothelial cell cultures, as assessed by endothelial cell markers. Over 90% of cultured rat heart cells stained for endothelial cell markers CD31 and von Willebrand Factor (data not shown).

To characterize sprouts ultrastructurally, we used confocal laser microscopy. Alexa Fluor 448 conjugated GSL I – IB$_4$ was used as a marker for endothelial cells, and Cy3-conjugated lectin anti-smooth muscle actin was used as a marker for smooth muscle cells and pericytes. A maximum intensity projection (Fig. 6A) of a bFGF-stimulated explant demonstrates the close interaction between the endothelial sprout and a smooth muscle cell or pericyte. The endothelial cells project filipodia into the fibrin gel (arrows). Volume rendering of the same data set indicate that the sprout appears solid, without a lumen (Fig. 6B).

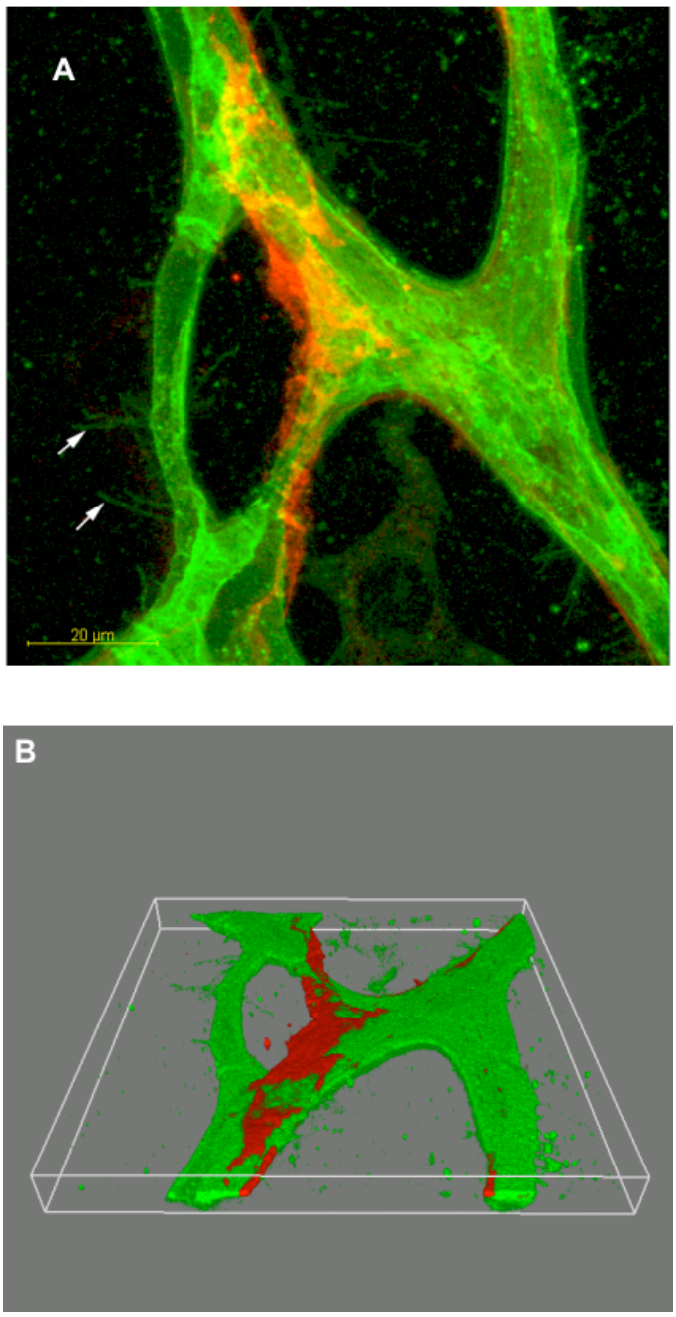

Fig. 6. Sprouting endothelial cells interact with smooth muscle cells or pericytes.
Heart pieces were stimulated with hrbFGF (10 ng/ml), and sprouts were stained after 10 days. Smooth muscle cells or pericytes (red, probed with Cy3-conjugated anti-smooth muscle actin) align themselves to endothelial sprouts (green, stained with Alexa Fluor 488 conjugated to GSL I – IB_4) Deconvolved confocal image stack.
A. Maximum intensity projection. Arrows point to filopodia arising from the endothelial cells. Scale bar: 20 □m
B. Volume rendering of the same data stack showing cell interactions. Note solid endothelial sprout (green) lack a lumen and interact with apposed smooth muscle cells (red). Dimensions of stack = 100 □m (x/y) and 10.5 □m (z).

To investigate myocardial tissue architecture and possible necrosis of heart tissue after 10 days of hypoxia, pieces of hearts were histologically analyzed (Fig. 7). In one of ten samples analyzed, necrosis could be detected in HE-stained tissue sections of PDGF-BB-stimulated hearts. However, in nine of ten samples analyzed, myocardial architecture was fairly well preserved, though cross-striations were faint and the spatial distribution of myocardial nuclei had changed (Fig. 7).

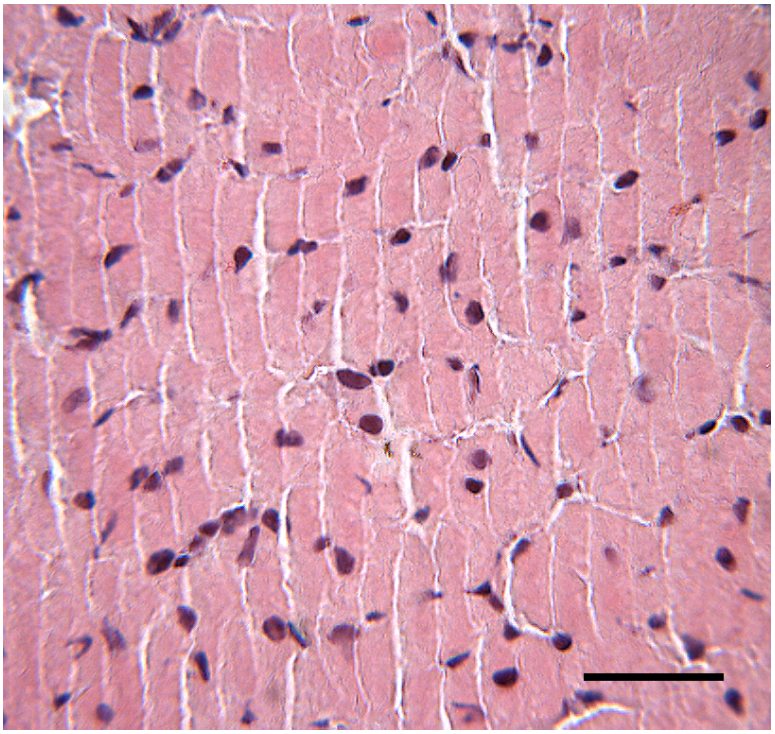

Fig 7. Most pieces of heart preserve myocardial architecture after 10 days of culture.
HE-stained section of a piece of mouse heart after 10 days of PDGF-BB stimulation under hypoxia in fibrin gels; myocardial architecture is fairly well preserved, though cross-striations disappeared and the spatial distribution of myocardial nuclei begins to change. Cell architecture resembles tissue as seen in ischemic myocard or after myocardial infarction (Bar = 50 μm).

Discussion

In this study we present a newly developed *in vitro* angiogenesis assay of the heart. Pieces of rat or mouse hearts are cultured in a three-dimensional fibrin gel matrix and outgrowing cells and sprouts are quantified and characterized. 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*.

We found that hypoxia and serum are required for angiogenesis to occur in the adult mouse or rat heart *in vitro*. FCS at a higher concentration (10%) to induced angiogenesis by itself. Thus, we used the lowest possible FCS concentration (5%) to avoid masking effects of growth factors by FCS. FCS contains numerous growth factors which in combination can synergize to induce angiogenesis [17].

Under hypoxia, but not under normoxia, *in vitro* angiogenesis sporadically occurred at a basal level in unstimulated heart cultures in the presence of 5% FCS. Under hypoxia, but not under normoxia, PDGF-BB, bFGF and $VEGF_{164}$ induced substantial angiogenesis *in vitro*. Among tested growth factors, PDGF-BB most efficiently stimulated *in vitro* angiogenesis. After 10 days of hypoxic culture, histology of PDGB-BB stimulated hearts showed a fairly well preserved architecture of cardiac tissue excluding possible necrosis. PDGF-BB-induced sprout formation could be repressed by application of low concentrations 10nM of rapamycin. Rapamycin specifically inhibits mTOR signalling and angiogenesis [9], [18]. mTOR has been shown to up-regulate hypoxia-inducible factor 1-a (HIF-1a) [19] and increase angiogenesis under hypoxia [9] and in tumors [20]. This effect of rapamycin in our assay shows the possibility to investigate signalling involved in growth factor-mediated heart angiogenesis and thus angiogenesis inhibitors *in vitro*.

Morphology of outgrowth differed upon administration of distinct growth factors. $VEGF_{164}$ mainly induced elongated sprouts with complex branching. PDGF-BB induced outgrowth of a mixture of organized branched endothelial sprouts, unorganized single endothelial cells and pericytes/smooth muscle cells, and bFGF induced mainly un-branched and elongated sprouts. Independent of the growth factor applied, sprouts were of endothelial origin. However, all growth factors also induced substantial outgrowth of non-endothelial cells that correlated with the extent of endothelial sprout formation. Thus, we have identified pure endothelial sprouts that are distinct depending on the stimulus; smooth muscle cells or pericytes start to align

along these sprouts, which is consistent with early vessel maturation. Similarly, in an *in vivo* model of tumour angiogenesis, pericytes were shown to attach to endothelial tubes at an early stage of sprout formation [21]. Nevertheless, it would be of great interest to extend the culturing period further to investigate smooth muscle cell pericytes attachment and thereby vessel maturation. However, our assay is time-limited to 10-12 days. Gels of positive controls (PDGF-BB-stimulated hearts) reach maximal and overgrowing sprout formation after 10 days of culture under hypoxia. Also, gels start to degrade after 12 days and it is not possible to carry experiments further.

Unlike later stages of angiogenesis *in vivo* continuous lumen is not present in the sprouts at the time point studied (data not shown). This might be due to the absence of blood flow and pressure in this assay of *in vitro* angiogenesis. Thus, physiologically this model may correspond to morphogenesis at an early stage of angiogenesis.

Importantly, rat endothelial cells from the gels can be further subcultured. Cells isolated were grown in medium enriched with endothelial cell supplement and, were of endothelial origin in 90% (data not shown). Isolated cells were then utilized for further experiments. Rat endothelial cells were kept in culture for maximally 14 passages. Unfortunately we were unable to subculture mouse heart cells for more than 2 passages.

Potent angiogenesis in response to PDGF-BB in the heart *in vitro* may surprise. Most clinical trials to improve angiogenesis in the heart focus on $VEGF_{164}$ and bFGF [1, 5, 22-26] and less on PDGF [8, 27]. However, a recent study has shown that PDGF limits the extent of myocardial infarction in a rat model via angiogenesis, and PDGF is angiogenic in rodents [8]. Other studies have also shown that PDGF is angiogenic *in vivo* and *in vitro* [28-30]. However, not much is known about mechanisms of PDGF-induced angiogenesis in the heart. PDGF may directly affect specific phenotypes of endothelial cells such as microvascular endothelial cells [29]. It may additionally induce expression of VEGF and other angiogenic growth factors with an endothelial specificity in non-endothelial cells and thus indirectly stimulate angiogenesis. In our assay we cannot exclude or confirm either of the two possibilities.

Age reduces the potential for angiogenesis [31-38]. Correspondingly, angiogenesis *in vitro* occurred earlier and was induced more easily in hearts of adolescent mice as compared with adult hearts. In few experiments with 20 weeks old mice, angiogenesis

was even more restricted than in the 12-week-old adult mice (data not shown). Age dependency of angiogenesis is in line with studies *in vivo* [31, 32, 34, 35] and *in vitro* [33, 36-38]. Angiogenesis allows normal tissue growth during development [39-42] and is necessary for organs and animals to mature. Specific physiologic and many pathologic processes reactivate angiogenesis [2, 39-41, 43]. However, the capacity for reparative mechanisms and angiogenesis decreases with age [31-35]. Thus, decreasing angiogenesis of the heart *in vitro* with age in our model is in line with corresponding observations *in vivo* [31-35]. Our results indicate that angiogenesis is restricted in the adult heart *in vitro* especially in comparison to other assays *in vitro* but still inducible by specific stimuli such as hypoxia and growth factors.

We compared our heart angiogenesis *in vitro* model to an aortic ring explant model which is a well-established and a widely used standard assay for angiogenesis *in vitro* [9, 16, 44-46]. Angiogenesis in heart and aorta differed slightly. *In vitro* angiogenesis of *aortae* also occurred under normoxia and in serum free culture conditions in contrast to *in vitro* angiogenesis of hearts. This indicates that angiogenesis may be regulated in an organ specific manner depending on culture and microenvironmental conditions. Similarly, others have reported angiogenesis to be regulated in an organ specific way and therefore claim a need for organ specific models [47, 48]. An alternative explanation of the differential findings in aortic versus heart assays may be that endothelial cells sprouting from a rodent aorta [9, 16, 44-46] are very likely of macrovascular origin. In contrast, endothelial cells sprouting from a small piece of heart are likely of microvascular origin, similar to angiogenesis *in vivo* [39]. Differences between the two assays may therefore also be due to micro- versus macrovascular origin of the endothelial cells. This hypothesis would be further confirmed by investigating the origin of sprouting cells in our *in vitro* assay. However, the characterization of sprouting cells in our assay is currently limited to the use of fluorochrome-conjugated antibodies and has to be further developed. The more restricted pattern of angiogenesis in the heart suggests a strong need to assess angiogenesis in a model that utilizes the organ of interest.

Experiments performed in rodents may not always be representative for humans. However, preliminary experiments with small pieces of explanted human left ventricle obtained from a heart transplant recipient displayed a similar angiogenic response *in vitro* (data not shown). Similar to rodent hearts a combination of growth

factors and hypoxia promoted sprout formation. Recently, a similar *in vitro* angiogenesis assay using samples of human cardiac auricle has been developed [49]. Comparable to our results $VEGF_{165}$ and FGF induced *in vitro* sprout formation by endothelial cells. In contrast to our results in mouse hearts, sprouts formed without addition of serum and under normoxic culture conditions. Differences between these two assays might be due to culture conditions, the difference in species, the different origin of the pieces from within the heart (left myocardium versus auricle), or other distinctions. In summary, the majority of our data obtained in rodents are in line with results obtained with human hearts [49]. Human tissue is not readily available and very often from diseased heart or parts of the heart of lesser interest for the question asked. Therefore, rodent hearts remain an alternative. Apart from availability, transgenic mice can be used to answer mechanistic questions in heart angiogenesis *in vitro*.

Obviously, our assay may not completely reflect myocardial angiogenesis *in vivo*. However, organ culture models may better simulate *in vivo* situations than other assays *in vitro* because they include surrounding non-endothelial cells in their microvascular environment. In addition, endothelial cells have not been pre-selected by passaging, are not in a proliferative state at the time of initiation of the experiment and may thus better represent a real-life situation [50, 51]. Hence, sprout formation in our assay could be mediated by direct stimulation of myocardial endothelial cells and possibly also by stimulation of surrounding hypoxic 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. Thus, 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*.

We have chosen a oxygen concentration of 21% O_2 for normoxia and 3% O_2 for hypoxia. However, we do not know the exact oxygen concentration in the piece of heart embedded in the culture gel. 21% and 3% O_2 represent oxygen concentrations in the incubator. Oxygen diffuses into the piece of heart and keeps most pieces viable for 10 days (see above). The effective oxygen concentrations to which the pieces and tissues of heart are exposed are lower in our experiments than the numbers given and

are not yet defined. To further investigate hypoxic oxygen concentrations we also investigated 1% O_2 on PDGF-stimulated hearts (data not shown). However, there were no differences in sprouting in response to PDGF-BB versus diluent between 1 and 3% O_2 (data not shown).

In conclusion, angiogenesis of the heart *in vitro* can be investigated with a simple assay. This assay allows a large series of experiments to be performed with a minimum of animals in a relatively short time. We have demonstrated the reliability of our model by reproducing known *in vivo* and *in vitro* phenomena. This model may allow to evaluate mechanisms of angiogenesis in hearts of normal, diseased and transgenic animals and to investigate substances that induce or repress angiogenesis in the heart.

Material and Methods

Animals

Experiments were performed with hearts and *aortae* of different mouse strains, mostly with OF1 mice. In addition, a few key experiments were repeated in black six mice to assess for potential inconsistencies amongst distinct mouse strains and in Sprague Dawley rats for inconsistencies across species. All hearts and aortae were obtained post mortem. The animals were euthanized and the hearts immediately transferred to PBS. Within half an hour after death the mouse hearts were embedded in fibrin gel (see below). To avoid damage to the heart tissue, hearts and aorta were consistently covered with PBS (also during fragmentation into small pieces). Mouse hearts and aortae were obtained from Dr. Bastian Hengerer at Novartis Basel (experiments registered and approved at the Veterinary Department of the city of Basel, ID 1687, experiment ID 20108). Rat hearts and aortae were obtained from the group of Prof. Peter Buser (experiments registered and approved at the Veterinary Department of the city of Basel, ID 791) and from the group of Prof. Stephan Krähenbühl (experiments registered and approved at the Veterinary Department of the city of Basel, ID 1744). Ages ranged from 4 to 20 weeks for mice and from 6 to 20 weeks for rats.

Assay of angiogenesis *in vitro*

Preparation and evaluation of the assay are similar for heart and aorta. The assay is based on the "three-dimensional" angiogenesis *in vitro* assay originally established by Nicosia and colleagues [16]. Briefly, gels were made by preparing a fibrin gel solution: 3 mg of Fibrinogen (Sigma-Aldrich, Buchs, Switzerland) per ml serum-free DMEM (Oxoid, Basel, Switzerland) with 0.1U/ml of thrombin (Sigma-Aldrich, Buchs, Switzerland) on ice. 100 µl/ well of this fibrin gel solution were immediately pipetted into each well of 48-well plates and allowed to polymerize for 30 min at 37°C. Gels were overlaid with 500 ml serum-free DMEM for at least 30 minutes. The medium overlaying the gel was removed and 0.5 - 1mm^3 cubes from the left ventricular myocardium of the heart or the aorta were placed onto the gels in each well and overlaid with 100 ml of fibrin gel solution. After 30 minutes of polymerization, gels were overlaid with 500 µl standard DMEM containing the

indicated concentration of fetal calf serum (FCS, Oxoid, Basel, Switzerland). Heart explants were then exposed to growth factors and incubated under normoxic (21% O_2) and hypoxic (3% O_2) conditions for the number of days indicated, with addition of fresh growth factors every second day: 10 ng/ml human recombinant (hr) PDGF-BB, 10 ng/ml, hr bFGF, 5 ng/ml, hr $VEGF_{164}$ (R&D systems, Minneapolis, MN); or without addition of growth factors (diluent). Growth factor concentrations were based on optimal doses for maximal proliferation in previously performed endothelial proliferation assays using rodent rat endothelial cells (data not shown). Rapamycin was added freshly 20 minutes before growth factors and included throughout normoxic and hypoxic incubations. The effective concentration of rapamycin (Alexis Corp (10 nM)) was established previously in mouse aortae [9]. Fibrin gels were protected from degradation by adding 300 µg/ml e-amino-caproic acid (Sigma-Aldrich, Buchs, Switzerland) every third 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). The extent of sprout formation was determined for each condition in octuplicates by comparison with a standardized scale (angiogenic index) by two independent investigators and averaged. The Angiogenic index was defined with the help of an image analysis software (AnalySIS Pro, Soft Imaging System, Gloor Instruments, Uster, Switzerland). The index is defined by the relation between the total area of outgrowing cells to the area of the embedded tissue in study (heart or aorta). Based on this a representative scale from 0 to 8 was defined.

Clear differentiations between agonists and antagonists can be seen after 7 days of incubation. After more than 12 days gel cultures start to disintegrate. Therefore the maximum incubation periods was set to 10 days.

Characterization of cells & tissue

For characterization of outgrowing cells and sprouts specific cell markers were directly applied to heart cultures in the gels. The contents of the well were fixed over night with 4% Paraformaldehyde (Merck AG, Dietikon, Switzerland), washed with PBS and incubated for 3 hours with specific cell markers: Fluorescein conjugated GSL I – IB4 (20 µg/ml; Rectolab S.A., Servion, Switzerland) for endothelial cells,

Cy3-conjugated anti □ smooth muscle actin (1:100; SMA; Fluka Chemie GmbH, Buchs, Switzerland) for smooth muscle cells or pericytes and Hoechst dye (Polysciences Europe GmbH, Eppelheim, Germany) for visualization of cell nuclei. After incubation with cell markers the probes were extensively washed with PBS.
For histological sections fibrin gel containing heart piece were transferred into a tube and fixed in 4% formalin overnight. Fixed tissue was embedded in paraffin and 2-4 µm sections were mounted, stained with haematoxylin-eosin (HE) and photographed digitally (ColorView II, Soft Imaging System, Gloor Instruments, Uster, Switzerland) on an inverted light microscope (Olympus IX50, Olympus, Schwerzenbach, Switzerland).

Confocal Laser Scanning Microscope Observation

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 specific cell markers: Alexa Fluor 448 conjugated GSL I – IB_4 (20 □g/ml; Molecular Probes, USA) for endothelial cells, Cy3-conjugated antibody anti- □ 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. Samples were mounted in FluorSave Reagent (Calbiochem) on glass slides and examined by confocal laser scanning microscopy using a confocal microscope (TCS 4D, Leica Microsystems, Glattbrugg, Switzerland) operating in the simultaneous acquisition mode. Image stacks were recorded using a planapochromatic 100x/NA 1.4 objective lens. Image stacks were recorded with a sampling density of 0.2 □m along the x- and y-axes and of 0.1 □m along the z-axis.
Image processing and restoration was performed with the Imaris software package (Bitplane AG, Zurich, Switzerland) equipped with the Huygens module (Scientific Volume Imaging BV, Hilversum, NL). Deconvolution was performed as previously described [52].

Statistical analysis

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

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iNOS is required for *in vitro* angiogenesis of hypoxic healthy mouse hearts

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Abstract

Nitric oxide (NO) is a key signaling molecule and regulator of angiogenesis. Inducible NOS (iNOS) expression significantly increases during myocardial ischemia and infarction, and iNOS modifies angiogenesis and vascular permeability but less than eNOS as assessed in models of tumor angiogenesis. In this study we investigated whether deficiency of iNOS affects myocardial angiogenesis under hypoxia *in vitro*.

Using three-dimensional-models of angiogenesis *in vitro*, we first investigated the effect of pharmacological NOS (L-NIO) and specific iNOS inhibitors (1400WT and SMT) on endothelial sprout formation of hearts and aortae from wild-type mice. In aortae, only SMT markedly affected sprouting in response to PDGF (-29%, $p < 0.05$) and VEGF (-52%, $p < 0.04$). However, in hearts all NOS inhibitors substantially decreased sprout formation.

In hearts of iNOS (-/-) mice, when compared to hearts from wild type mice, endothelial sprout formation did not occur at all under hypoxia and could not be rescued with VEGF, FGF or PDGF. In contrast, endothelial sprout formation still occurred, though to a reduced extent (-40% for diluent, -63% for PDGF, -61% for FGF and –48% for VEGF, all $p < 0.05$), in pieces of aortae from the same iNOS (-/-) mice when compared to aortae from wild type mice.

In conclusion, mouse heart vessels require iNOS for angiogenesis *in vitro* to occur whereas angiogenesis *in vitro* originating from other blood vessel types such as the aorta may depend less on iNOS.

Introduction

Ischemia and tissue hypoxia of the myocardium occurs as a consequence of coronary heart disease or left ventricular remodeling in hypertension. Ischemia elicits vasodilation and angiogenesis, which serve to reduce tissue hypoxia.

Nitric oxide (NO) is an important signaling molecule that regulates physiological processes such as neurotransmission, immune defense, cell death, vascular permeability and vasodilatation in many tissues [1]. NO also modulates angiogenesis, being required for endothelial cell proliferation and migration *in vivo* and *in vitro* [2, 3]. Thus, angiogenesis *in vivo* and growth-factor-mediated endothelial tube formation *in vitro* require NO biosynthesis [3-8]. NO also enables growth regulatory molecules such as VEGF and bFGF to induce angiogenesis [3, 9, 10]. We have previously shown that Fibroblast Growth Factor (bFGF)-mediated angiogenesis requires NO in the mesenteric vascular bed of normal and portal hypertensive rats *in vivo* [11, 12]. Some of these angiogenic growth regulatory molecules in turn can trigger NO-synthases (NOS): VEGF increases permeability and decreases vascular tone by activating endothelial NOS (eNOS) [13, 14]. Both inducible NOS (iNOS) and (eNOS) (-/-) mice display impaired angiogenesis in a model of skin flap survival and in the ischemic hindlimb, respectively [15-17]. eNOS plays a significant role during reparative angiogenesis [18]. However, iNOS expression has been more extensively described in tumor angiogenesis were it was found to be increased and its levels correlated to vascularization [3-5]. To our knowledge, the role of iNOS on heart angiogenesis has neither been studied *in vivo* nor *in vitro* so far. In this study we therefore aimed at assessing the specific role of iNOS in angiogenesis of the heart *in vitro* in comparison to another tissue under hypoxia. We have used a recently developed *in vitro* model of angiogenesis in the heart [19] in comparison to a model of angiogenesis *in vitro* from aortae and investigated endothelial sprout formation in response to angiogenic growth regulatory molecules in wild type mice and in iNOS (-/-) mice.

Material and Methods

Animals

Experiments were performed with hearts and aortae of 8 to 12 week OF1 and iNOS (-/-) mice (Strain: B6;129P2-Nos2tm1Lau/J, The Jackson Laboratory, Maine,-USA), obtained and used immediately post mortem. Organs were received from Dr. Christophe von Garnier at the Department of Research (University Hospital Basel) (experiments registered and approved by the Veterinary Department of the city of Basel).

Assays of angiogenesis *in vitro*

Pieces of heart [19] and aorta [20] were cultured inside a fibrin gel as described previously. Explants were exposed to growth factors and incubated under moderate hypoxia (3% O2) for 10 days, with addition of fresh growth factors every second day: 10 ng/ml human recombinant Platelet-derived Growth Factor-BB (PDGF-BB), 5 ng/ml rat recombinant Vascular Endothelial Growth Factor 164 (VEGF164) or 10 ng/ml human recombinant basic Fibroblast Growth Factor (bFGF) (all from R&D systems, Minneapolis, MN). Endothelial sprouts were then photographed and analyzed as described previously [19].

Inhibitors were included from initiation of the experiment and throughout hypoxic incubation. 100 M of inhibitors were added freshly each time 20 minutes before addition of growth factors. The following inhibitors were used: SMT (S-Methylisothiourea), L-NIO (L-N5-(1-Iminoethyl)-ornithine) and 1400W (N-(3(Aminomethyl)benzyl)acetamidine) (all from Alexis Corp., Lausen, Switzerland).

Statistical Analysis

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

Results

We have shown previously that angiogenesis *in vitro* of the heart of wild type mice takes place only under hypoxia (1%-3% O_2) and is amplified further by different angiogenic growth regulatory molecules [19]. We have therefore performed all assays of angiogenesis *in vitro* under conditions of moderate hypoxia (3% O_2). To investigate and compare whether results are specific for angiogenesis in the heart, we also investigated *in vitro* angiogenesis in another tissue in parallel, namely aortic explants from wild type and iNOS (-/-) mice.

To study the role of iNOS in angiogenesis of the heart we first included pharmacological NOS inhibitors in assays of angiogenesis *in vitro* of wild-type hearts and aortas. We focused on three NOS inhibitors; L-NIO, a general NOS inhibitor, and two selective inhibitors of iNOS; SMT and 1400W. Under hypoxia the different NOS inhibitors distinctly affected *in vitro* sprouting of aortae when compared to hearts (Fig. 1).

In aortae, L-NIO and 1400W had very little effect on sprouting induced by PDGF and VEGF, but SMT decreased sprouting induced by PDGF (- 29%, $p < 0.05$) and VEGF (- 52%, $p < 0.04$) (Fig. 1A). In the heart, L-NIO and SMT significantly decreased the levels of sprouting induced by PDGF (-35% and –51%, respectively, both $p < 0.05$) (Fig. 1B). VEGF-induced angiogenesis *in vitro* was reduced by all NOS inhibitors; L-NIO (-45%, $p < 0.04$), 1400W (-39%, $p < 0.04$) and SMT (-40%, $p < 0.04$) (Fig. 1B). Thus, the effects of iNOS inhibition on angiogenesis *in vitro* were more pronounced in mouse hearts than in mouse aortae.

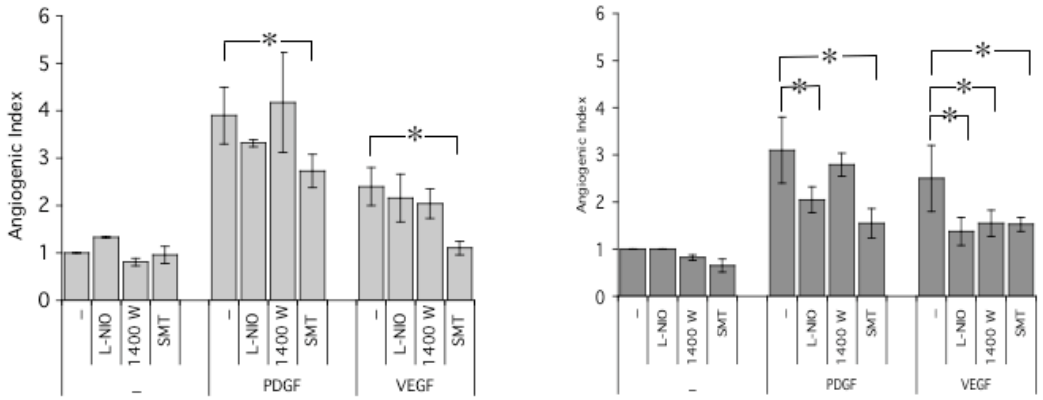

Figure 1. Pharmacological inhibitors of NOS reduce growth factor-induced *in vitro* angiogenesis more in the heart (B) than in the aorta (A)

In vitro angiogenesis was quantified after 10 days in hypoxia and after addition of growth regulatory molecules: PDGF-BB (10ng/ml) and $VEGF_{164}$ (5ng/ml) combined with 100 □mol/L (□M) of pharmacological NOS inhibitors: 1400W, SMT and L-NIO. A standardized scale ranging from 0 to 6 indicates the degree of sprouting (angiogenic index). Data points given represent the mean of three experiments ± SEM. * = $p < 0.05$ (Mann-Whitney Test) indicates significant difference to equivalent untreated controls.

To corroborate these findings, we studied the role of iNOS in heart angiogenesis *in vitro* of wild type and iNOS (-/-) mice. In aortas of wild type animals PDGF-BB, bFGF and VEGF induced angiogenesis *in vitro* (Fig. 2A). In aortas of iNOS (-/-) animals sprout formation was clearly reduced in all conditions but still present (Fig. 2A). Endothelial sprout formation in aortas of iNOS (-/-) aortae was reduced by 40% for diluent in comparison to in comparison to aortas of wild-type animals ($p < 0.05$), 48% for VEGF ($p < 0.05$), 61% for FGF ($p < 0.05$) and maximally by 63% for PDGF ($p < 0.05$). In hearts of wild type mice, PDGF, bFGF, and VEGF induced substantial angiogenesis *in vitro* as described before (Fig. 2B) [19]. In contrast, *in vitro* angiogenesis of the heart in iNOS (-/-) mice was totally abrogated. None of the growth factors tested induced any endothelial sprout formation (Fig. 2B).

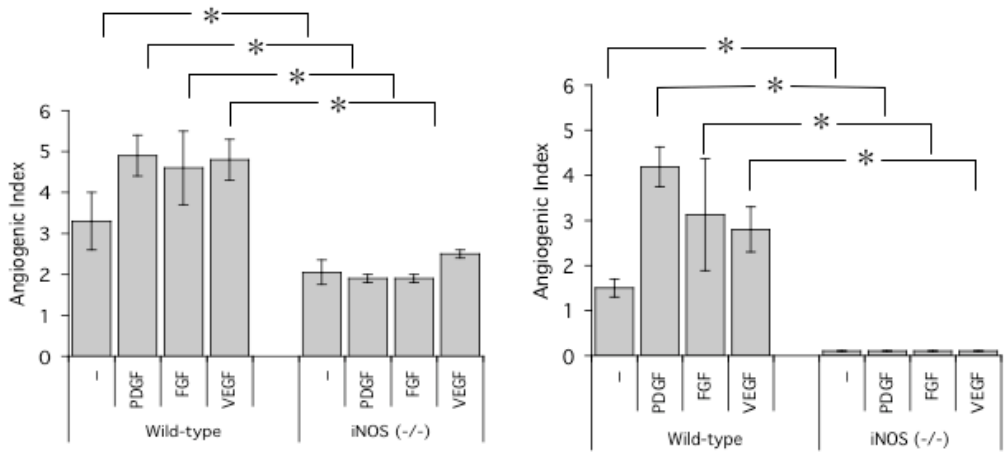

Figure 2. *In vitro* angiogenesis of the heart is abrogated under hypoxia in iNOS (-/-) mice.

In vitro angiogenesis of aortae and hearts 10 days after addition of growth regulatory molecules: PDGF-BB (10ng/ml), bFGF (10 ng/ml) and $VEGF_{164}$ (5ng/ml). A standardized scale ranging from 0 to 6 indicates the degree of sprouting (angiogenic index). Data points given represent the mean of three experiments ± SEM. * = $p < 0.05$ (Mann-Whitney Test) indicates significant difference to equivalent wild type samples.

Discussion

In this study we have investigated whether iNOS is required for angiogenesis *in vitro* of the heart versus aortae. *In vitro* angiogenesis of the heart in iNOS (-/-) mice under hypoxia was totally abrogated and endothelial sprout formation could not be restored with angiogenic growth factors VEGF, FGF or PDGF. We obtained similar results with pharmacological NOS inhibitors. Most of the used NOS inhibitors, particularly the iNOS-specific inhibitor SMT, substantially reduced but did not fully abrogate sprouting in wild type hearts. The quantitative differences observed amongst the different NOS inhibitors are probably due to their isoform specificity and their particular mechanism of NOS inhibition. In Fig. 1B we observe that 1400W can inhibit sprouting induced by VEGF but not by PDGF. This may suggest that PDGF might activate pathways leading to endothelial sprout formation that are less dependent on iNOS. PDGF-induced intracellular signals encompass a much broader spectrum [21] than those in response to VEGF [22]. Furthermore, VEGF receptor expression is restricted almost exclusively to vascular endothelial cells [23], while PDGF receptors can be found in all connective tissue cells and certain other cell types including endothelial cells [21, 24, 25]. Taken together our results in iNOS (-/-) mouse hearts and with pharmacological NOS inhibitors suggest a central role for iNOS in allowing angiogenesis of the heart.

Experiments in rabbits have shown that iNOS in cardiac tissue is expressed in response to various physiological and pathogenic factors only. These include myocardial infarction and inflammation [26]. Recent evidence, however, localizes iNOS to neonatal and adult cardiomyocytes, to the myocardium of adult rats, and the myocardium of patients with dilative cardiomyopathy [27]. Moreover, a cardioprotective role of iNOS has been confirmed; iNOS (-/-) mice are more vulnerable to death after reperfusion and to myocardial injury associated with myocardial ischemia and reperfusion injury than wild type mice [28]. Hypothetically, decreased viability of iNOS (-/-) mice may also be due to impaired angiogenesis. Angiogenesis is one mechanism that improves collateral circulation and thus blood supply to ischemic areas. However, angiogenesis requires prolonged ischemia and a few days to occur. Similar to the situation *in vivo* first sprout formation occurs after ~3 days in our *in vitro* assay, correlating with the time point of late cardiac

preconditioning [29]. Our results support an important role for iNOS in heart angiogenesis. iNOS may therefore protect from tissue ischemia via angiogenesis.

In addition, the requirement of iNOS in heart angiogenesis also suggests that angiogenesis is regulated in an organ specific way. *In vitro* angiogenesis of the heart was totally abrogated in iNOS (-/-) mice. In contrast, *in vitro* angiogenesis in aortas was still present, albeit at a reduced rate and without a response to angiogenic growth factors. This may be due to phenotypic differences of endothelial cells of large vessels such as the aorta versus the cardiac microvasculature [30] from which endothelial cells sprout to form new vessels. Thus, iNOS-independent pathways may exist that can regulate angiogenesis in aortae and potentially many other organs and that are not present or less active in the hypoxic heart. This supports the need of organ specific models for angiogenesis [31] and the concept that the angiogenic response in adult hypoxic hearts is far more restricted than in other organs.

Our experiments leave many questions unanswered. Effects of eNOS on angiogenesis of the heart have not been investigated in this study and cannot be excluded. Other experiments measuring myocardial capillary densities in eNOS knock out animals have shown that deficiency in eNOS impairs myocardial angiogenesis [32]. The use of NO donors to restore angiogenesis has not been examined. The role of iNOS on angiogenesis of the heart in diseased hearts and in hearts of other species also remains to be studied.

In conclusion, we have shown that *in vitro* angiogenesis of the heart under hypoxia requires iNOS. Furthermore, we have shown an organ-specific requirement of the heart for iNOS, i.e., that angiogenesis is more restricted in a model *in vitro* of the adult hypoxic heart than in another model based on the aorta and that angiogenesis is regulated in organ-specific ways.

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Angiotensin II induces angiogenesis *in vitro* through an AT2 – BKR2 pathway in the hypoxic mouse heart.

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Abstract

Introduction: Arterial hypertension (AH) is characterized by increased vasoconstriction through activation of the Renin-Angiotensin-Aldosterone System (RAAS), but also by micro-vascular rarefaction in the heart, leading to myocardial hypoxia. Hypoxia triggers angiogenesis that serves to relieve ischemia. Angiotensin II (Ang II) has been reported to induce formation of new microvessels. This study aimed to define the role of AT receptor subtypes in Ang II-mediated angiogenic effects of the heart under hypoxia *in vitro.*

Results: Endothelial sprout formation from hearts of 12-14 week old wild-type B6 mice was assessed under normoxia (21% O_2) and hypoxia (3% O_2) during a 7-day period of *in vitro* culture. Under hypoxia but not under normoxia, Ang II dose dependently induced significant endothelial sprout formation. Maximal growth of Ang II-treated hypoxic hearts was observed at 100 nM of Ang II (1.9 times of control, $p<0.05$). AT1 blockade by Losartan did not affect Ang II-induced sprouting in the heart of wild-type mice. Conversely, AT2 antagonist (PD 123319) abrogated this response (-35%, $p<0.05$). In hearts from AT1 knockout mice, Ang II elicited angiogenic response was preserved (1.9 times of control, $p<0.05$) and could also be blocked by PD 123319 (-47%, $p<0.05$). In contrast, Ang II did not induce sprouting from hearts of AT2 knockout mice. Interestingly, Ang II-mediated angiogenesis could be abrogated with a specific Bradykinin receptor 2 (BKR2) inhibitor (HOE140) in both wild type and AT1 knockout animals (-66% and –67% respectively $p<0.05$). Finally, Ang II did not induce endothelial sprout formation in hearts from BKR2 knockout mice in contrast to wild type controls.

Conclusion: The results suggest a pro-angiogenic role of Ang Iiin the hypoxic heart *in vitro*. The response to Ang II occurred only under hypoxia. Under these conditions, Ang II induced angiogenesis via the AT2 receptor and interestingly the BKR2, suggesting that the AT2 receptor activates the BKR2 via a yet unknown mechanism to mediate angiogenesis.

Introduction

Angiotensin II is a key regulator of blood pressure and a main effector peptide of the Renin Angiotensin Alsoterone system (RAAS). The RAAS and its components are targeted to treat hypertension in humans [1]. Hypertension can lead to left ventricular hypertrophy and ischemic heart disease; these conditions are characterized by insufficient blood supply to the myocardium. This leaves vital myocardial areas ischemic and impairs myocardial function. Myocardial angiogenesis, i.e., the growth of new microvessels, is one of the endogenous protective responses to counteract tissue hypoxia.

Interestingly, Ang II tends to restore blood flow in the setting of acute ischemia in mice [2]. This may be due to the activation of pre-existing collateral vascular pathways. Moreover, Ang II is angiogenic in animal models of hind limb ischemia [2] and the rabbit cornea angiogenesis assay [3]. However, it is unclear how and under which conditions Ang II might act as an angiogenic molecule in the heart; At least two distinct Ang II receptor subtypes (designated subtypes 1 and 2) have been identified on the basis of their difference in structure and pharmacological and biochemical properties [4, 5]. Both Ang II receptor subtypes, AT1 and AT2, are expressed in the heart [6]. It is through the activation of the AT1 receptor, that Ang II exerts many of its well established cardiovascular effects, including vasoconstriction, sodium retention, aldosterone secretion and trophic effects [7]. The AT1 receptor is expressed ubiquitously. By contrast, the physiological role of the AT2 receptor remains to be fully defined. The AT2 receptor is constitutively expressed at low densities in the adult in adrenal glands, vascular endothelial cells, kidneys, uterus, ovarian granulosa cells and some brain areas [7]. AT2 receptor is upregulated in cardiac tissue in response to ischemia and inflammation [8]. A number of different experimental models, suggest that the mechanism underlying some AT2 receptor functions may involve the release of endothelial-derived vasodilators, nitric oxide (NO) and Bradykinin (BK) [9-11]. BK has been shown to promote angiogenesis via its BKR2 by increasing vascular and endothelial permeability and by up-regulating of VEGF via BKR2 [12]. Under some specific circumstances such as ischemia and myocardial hypoxia, the AT2 receptor may thus counterbalance the vasoconstricting effects of the AT1R.

In the present study we have investigated the angiogenic capacity of Ang II in an *in vitro* model of angiogenesis of the rodent heart under conditions of normoxia (21%

O_2) and severe (1% O_2) hypoxia. We aimed to better define the role of AT receptor subtypes in Ang II-mediated angiogenic effects by using selective pharmacological agonists and antagonists as well as knockout mice for the various receptors. Furthermore, we have examined the involvement of Bradykinin receptor 2 as a possible effector downstream of AT receptor activation.

Results

Angiotensin II induces sprouting of the adult mouse heart under hypoxia.

We first tested the effect of Angiotensin II (Ang II) in an *in vitro* model of angiogenesis in the heart both under normoxia (21% oxygen) and hypoxia (1% oxygen). Under conditions of normoxia neither Ang II, nor the angiogenic growth factor VEGF [13] that was used as positive control elicited a significant response (Fig 1a and 1b). Under hypoxia both VEGF and Ang II elicited a significant response when compared to their normoxic controls (Fig 1a and 1b). Both these responses were of similar magnitude (2,2 and 2,4 fold increase respectively, when compared to negative control $p<0.05$) (Fig 1a and 1b). To characterize the cells involved in Ang II-induced sprout formation, fluorescence-labelled cell markers were directly applied on the fibrin gel cultures (Fig. 1a insert). These stainings showed sprouts of endothelial cells and, aligned to them smooth muscle cells/pericytes.

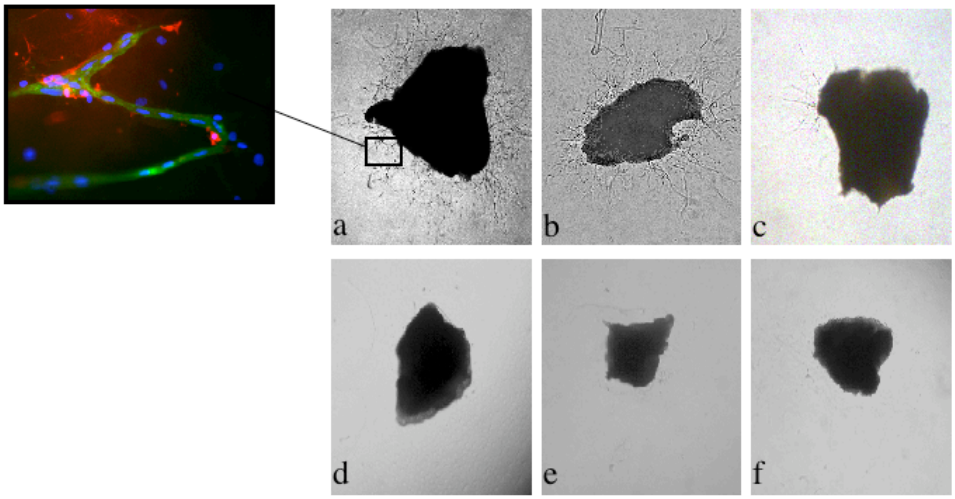

Figure 1a: Heart Angiogenesis Assay.

Mice heart after 7 days in culture in 1% oxygen (a, b, c) or 21% oxygen (d, e, f). Stimulated with 100nM angiotensin II (a,d); stimulated with 10ng/ml VEGF (b, e) or untreated (c, f). Magnification: 40X. Insert:Endothelial cells are visualized by Fluorescein conjugated GSL I – IB_4 (green) and pericytes or smooth muscle cells by Cy3 conjugated SMA (red), nuclei were stained with Hoechst (blue).

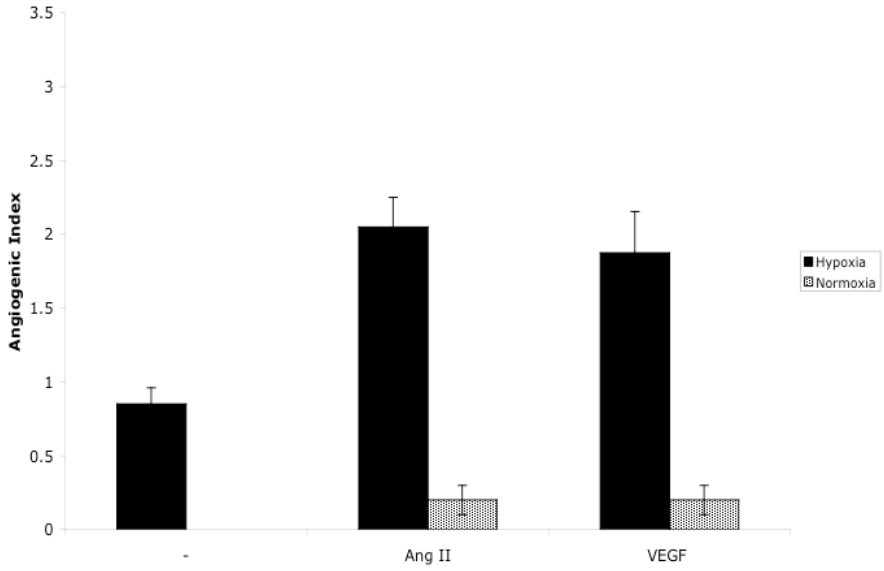

Figure 1b: Angiotensin II induces sprouting in adult mouse hearts in hypoxia.
Angiotensin II (100 nM) or VEGF164 (10 ng/ml) were added to the culture medium containing 5% FCS under hypoxia (1% oxygen) or normoxia (21%) for 7 days. Ang II and VEGF induced sprouting to a similar extent. Hypoxia significantly increased sprouting in all experimental conditions in comparison to normoxia and in hypoxia Ang II and VEGF induced significant sprouting with respect to control (*$p<0.05$ Mann-Whitney test). A standardized scale raging from 0 to 4 indicates the degree of cellular outgrowth (angiogenic index). Data points represent the mean of 5 independent experiments + SEM.

Since hypoxia is a prerequisite for *in vitro* angiogenesis of the heart (see above and [14]) we performed all consecutive experiments under hypoxia. To study the potency of Ang II as an angiogenic factor we stimulated the pieces of hearts with a wide range of concentrations of Ang II (100pM to 1mM). Only high concentrations of Ang II, i.e. above 1nM to 1uM, induced angiogenesis under hypoxia (Figure 1c). Lower concentrations of Ang II down to 1pM were also tested but did not induce a significant angiogenic response (data not shown). Therefore we concluded that Ang II dose dependently induces endothelial sprouting in the adult mouse heart under hypoxia with a maximum at a concentration of 100nM.

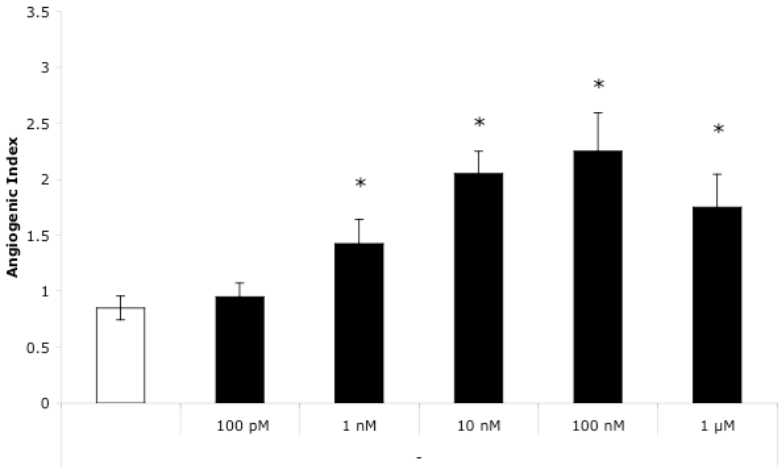

Figure 1c: Angiotensin II induces dose dependent sprouting in adult moue hearts in hypoxia.
Angiotensin II (concentrations ranging from 1pM to 1uM) added to the culture medium containing 5% FCS under hypoxia (1% oxygen) for 7 days. A standardized scale raging from 0 to 4 indicates the degree of cellular outgrowth (angiogenic index). Low doses of Ang II did not induce significant sprouting but higher doses do, showing a clear dose dependency (*$p<0.05$ Mann-Whitney test). Data points represent the mean of 5 independent experiments + SEM.

Angiotensin II induces sprouting through the AT2 receptor.

Next, we determined, the AT receptor subtypes which are required for Ang II-induced spout formation. The specific AT2 agonist CGP-42112 induced an angiogenic response very similar to that observed when hearts were stimulated with Ang II (2 fold increase with 100nM CGP-42112) (Fig 2a).

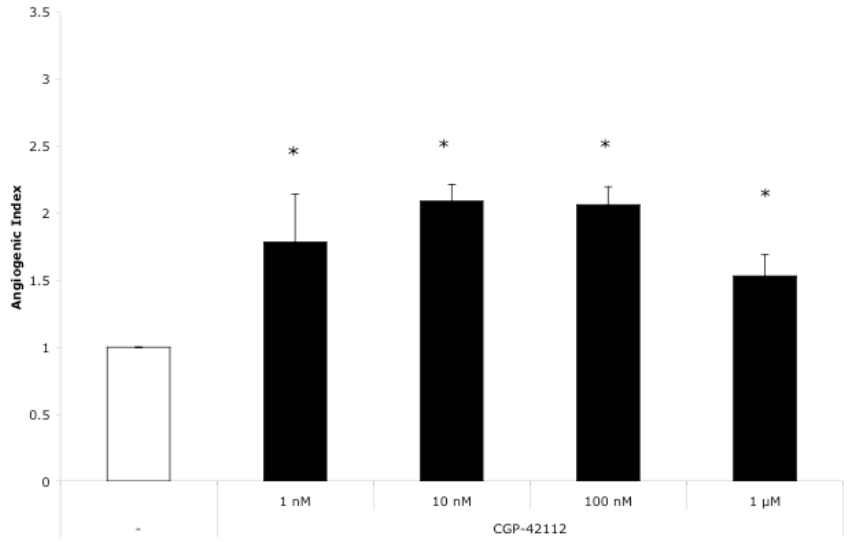

Figure 2a: The AT2 agonist, CGP-42112, induces dose dependent sprouting in adult mouse hearts in hypoxia.
CGP-42112 (concentrations ranging from 1nM to 1mM) added to the culture medium containing 5% FCS under hypoxia (1% oxygen) for 7 days. A standardized scale raging from 0 to 4 indicates the degree of cellular outgrowth (angiogenic index). CGP-42112 induced significant sprouting at all concentrations tested in a dose dependent way (*$p<0.05$ Mann-Whitney test). Data points represent the mean of 5 independent experiments + SEM.

CGP-42112 induced a dose dependent response with a peak at 10nM-100nM suggesting that the AT2 receptor might mediate Ang II induced angiogenesis. To further corroborate this finding pieces of hearts were stimulated with Ang II and with pharmacological AT receptor inhibitors (Fig 2b). Losartan, an AT1 inhibitor, did not have an effect on sprout formation induced by Ang II under hypoxia. On the other hand, PD 123319, an AT2 antagonist, significantly reduced (-35%, $p<0.05$) the level of sprouting induced by Ang II. When a combination of both antagonists was used the response observed was very similar to that seen with PD 123319 alone (-40%).

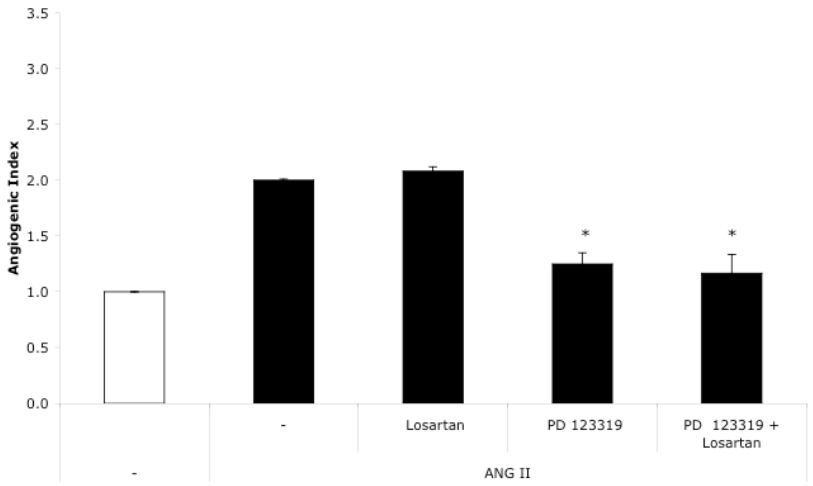

Figure 2b: Blockade with AT2 antagonist, PD 123319, abrogates Ang II-induced sprouting but blockade with AT1 antagonist, Losartan, does not.
Angiotensin II (100nM) alone or in combination with Losartan (1mM) and/or PD 123319 (1mM) added to the culture medium containing 5% FCS under hypoxia (1% oxygen) for 7 days. A standardized scale raging from 0 to 4 indicates the degree of cellular outgrowth (angiogenic index). Blockade with PD 123319 abrogates growth induced by Ang II (*$p<0.05$ Mann-Whitney test). Blockade with losartan did not induce a significant reduction in outgrowth induced by Ang II (p=ns). Data points represent the mean of 5 independent experiments + SEM.

Also, CGP-42112-induced sprout formation could be inhibited by AT2 receptor blocker (PD123319) but not by AT1 receptor blocker (Losartan)(data not shown). Taken together, these results confirm the importance of AT2 receptor in mediating angiogenesis of the adult mouse heart under hypoxia.

Angiotensin II does not induce sprouting in AT2 knockout animals.

In order to confirm these results we used hearts from different knockout animals. Sprout formation was absent in hearts derived from adult AT2 knockout animals (Fig 3a) when stimulated with Ang II in hypoxia. The same results were obtained when the AT1 receptor was blocked with Losartan. Stimulation of AT2 knockout hearts with VEGF induced a significant level of sprouting when compared to controls (2.6 fold increase, $p<0.05$) suggesting, that angiogenesis *in vitro* can still be induced via mechanisms alterative to Ang II.

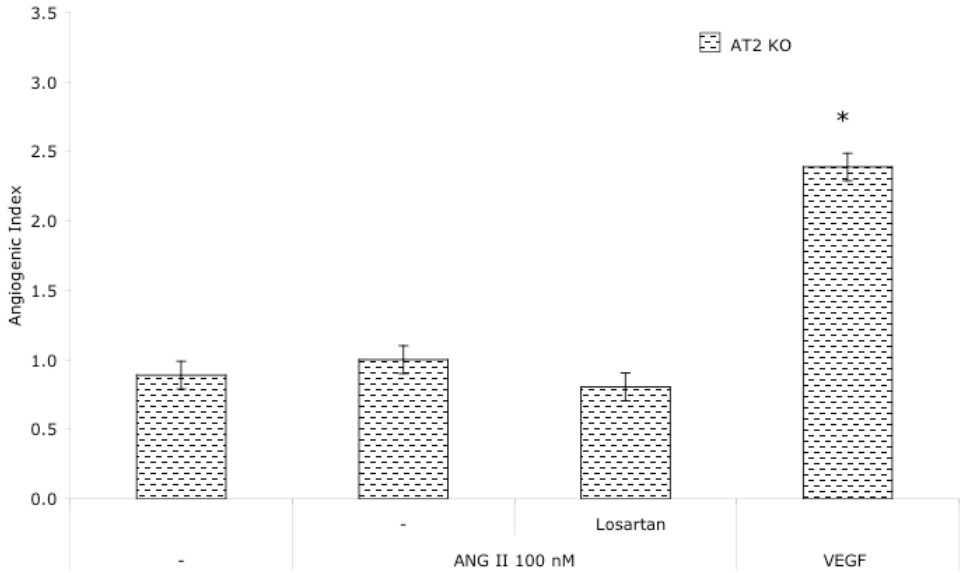

Figure 3a: Angiotensin II does not induce sprouting in AT2 knockout mice in hypoxia. Pieces of hearts from AT2 KO mice stimulated with angiotensin II (100nM) alone or in combination with Losartan (1 mM) or VEGF164 (10 ng/ml) were added to the culture medium containing 5% FCS under hypoxia (1% oxygen) for 7 days. A standardized scale raging from 0 to 4 indicates the degree of cellular outgrowth (angiogenic index). Angiotensin II could not induce a significant level of sprouting and blockade with Losartan did not change this effect (*p=ns Mann-Whitney test), VEGF: *p<0.05 Mann-Whitney test. Data points represent the mean of 5 independent experiments + SEM.

On the other hand, Ang II induced sprouting in hearts from adult AT1 knockout animals (1.9 fold increase, $p<0.05$) under hypoxia (Fig 3b). This sprouting was inhibited by PD 123319 (-47%, $p<0.05$), the inhibitor of the AT2 receptor, but not by Losartan. Thus, the receptor mediating the angiogenic response *in vitro* to Ang II in the heathy adult hypoxic mouse heart is the AT2 receptor.

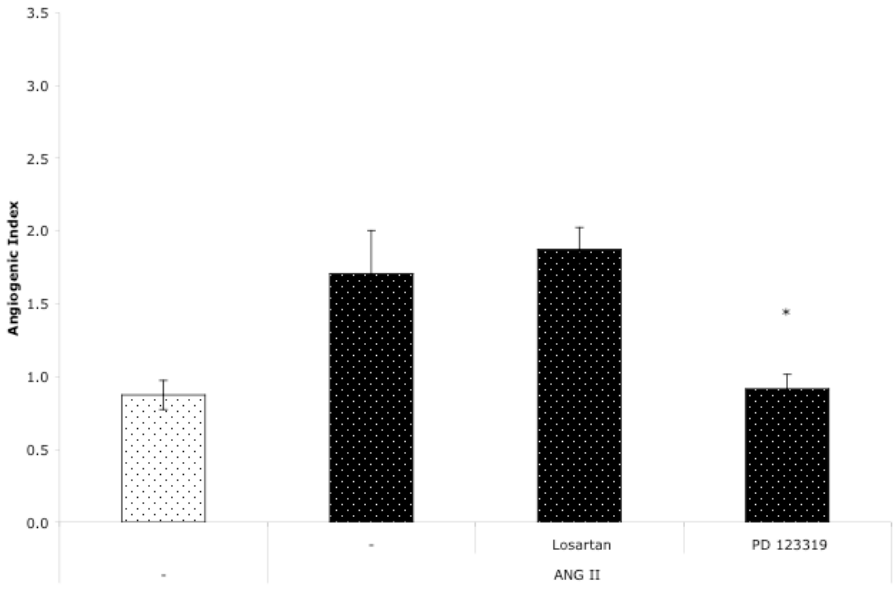

Figure 3b: Blockade with AT2 antagonist, PD 123319, abrogates Ang II-induced sprouting in AT1 KO mice in hypoxia
To pieces of hearts from AT1 KO mice, angiotensin II (100nM) alone or in combination with Losartan (1mM) and or PD 123319 (1mM) added to the culture medium containing 5% FCS under hypoxia (1% oxygen) for 7 days. A standardized scale raging from 0 to 4 indicates the degree of cellular outgrowth (angiogenic index). Blockade with PD 123319 abrogates growth induced by Ang II (*$p<0.05$ Mann-Whitney test). Data points represent the mean of 5 independent experiments + SEM.

Angiotensin II induces sprouting via AT2 –BKR2 pathway

The AT2 receptor may interact with bradykinin receptor 2 (BKR2) as part of it's signalling pathway [15]. To study the role of the BKR2 in angiogenesis induced by Ang II we stimulated hypoxic adult mouse hearts *in vitro* with Ang II in both wild-type and AT1 knockout animals in the presence of HOE 140, a specific BKR2 antagonist (Fig 4a).

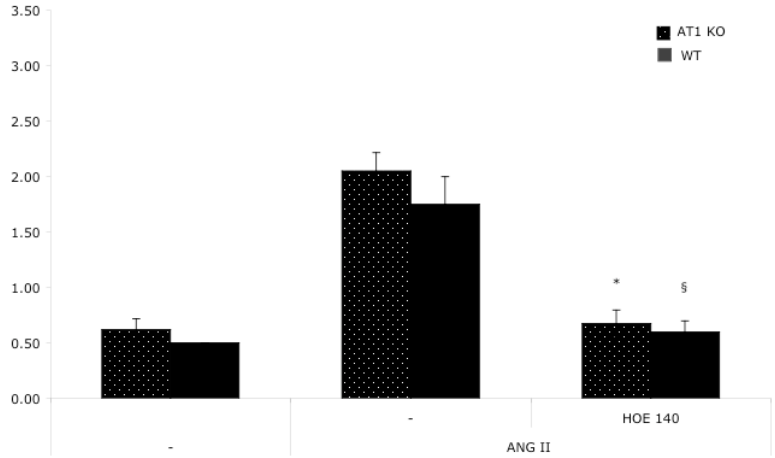

Figure 4a: Blockade with BKR2 antagonist, HOE 140, abrogates Ang II-induced sprouting in wild type and AT1 KO mice, in hypoxia.
Pieces of hearts from wild type or AT1 KO mice stimulated with angiotensin II (100nM) alone or in combination with HOE 140 (100 nM) added to the culture medium containing 5% FCS under hypoxia (1% oxygen) for 7 days. A standardized scale raging from 0 to 4 indicates the degree of cellular outgrowth (angiogenic index). Blockade with HOE 140 abrogates growth induced by Ang II in both AT1 KO and wild type mice (§ $p<0.05$ Mann-Whitney test or *$p<0.05$ Mann-Whitney test). Data points represent the mean of 5 independent experiments + SEM.

In hearts from both wild type and AT1-KO animals, Ang II induced a clear angiogenic response (wt: 3.5 fold increase, AT1-KO: 3.3 fold increase, $p<0.05$ for both) that was abrogated by HOE 140 (-66% and –67% respectively, $p<0.05$). To further corroborate the finding that Ang II-mediated angiogenesis requires BKR2 experiments were repeated in BKR2 knockout animals (Fig 4b). Neither Ang II nor VEGF induced a significant level of sprouting in BKR2 knockout mice.

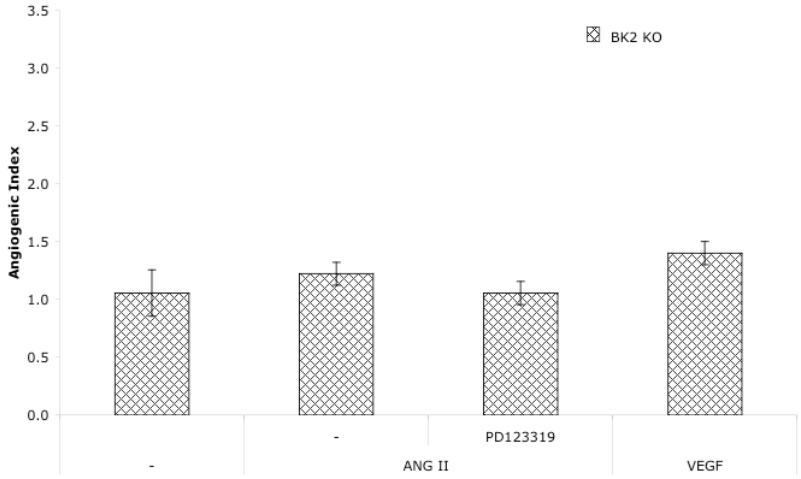

Figure 4b: Angiotensin II does not induce sprouting in BK2 KO mice.
Pieces of hearts from BK2 KO mice stimulated with angiotensin II (100nM) alone or in combination with PD123319 (1 uM) or VEGF164 (10 ng/ml) were added to the culture medium containing 5% FCS under hypoxia (1% oxygen) for 7 days. A standardized scale raging from 0 to 4 indicates the degree of cellular outgrowth (angiogenic index). Angiotensin II could not induce a significant level of sprouting and blockade with PD 123319 did not change this effect (*p=ns Mann-Whitney test). VEGF: *p<0.05 Mann-Whitney test. Data points represent the mean of 5 independent experiments + SEM.

We conclude that Ang II is angiogenic in the healthy adult mouse heart under hypoxia and that this response is mediated via a pathway involving both theAT2 receptor and the BKR2.

Discussion

Our results demonstrate conclusively that Ang II induces angiogenesis *in vitro* of the healthy mouse heart under hypoxia, but not under normoxia. This response is dose dependent with a peak at 100 nM. Furthermore, the angiogenic response requires the AT2 receptor and the BKR2 , but not the AT1 receptor. The data obtained are based both on the usage of pharmacological agonists and antagonists of Ang II / BK receptors and hearts of corresponding receptor knockout mice. Blockade of AT1 receptor preserves cardiac function after myocardial infarction by inhibiting AT1 receptor mediated pathways [16]. In addition, blockade of AT1 receptor pathways may also uncover beneficial effects of Ang II via the AT2 receptor. The results of our studies suggest that such beneficial effects may also be due to the stimulation of AT2 receptor culminating in angiogenesis.

The role of AT2 receptor in angiogenesis is not yet fully investigated. Microvessel density was decreased after myocardial infarction in transgenic rats overexpressing the AT1 receptor, and reverted by the use of Losartan [17]. Furthermore, Ang II-induced angiogenesis observed in alginate implants is linked to the AT2 receptor; impaired induction of angiogenesis was obtained in AT2 receptor knockout mice [18]. In our study Ang II and the AT2 agonist CGP-42112 induce angiogenesis of the heart during hypoxia. This response was abrogated by HOE 140, a BKR2 inhibitor. In line with this, Ang II was not able to induce angiogenesis in BK2 knockout mice. This points at an important role of the BKR2 in angiogenesis mediated by Ang II. These findings for the first time lend experimental support for the proposed mechanism, that the AT2 receptor might exert downstream effects via the BK2 receptor [15]. Experimental evidence that BKR2 activation leads to angiogenesis, however, is increasing. The proangiogenic effect of ACE inhibition is mediated by the BKR2 pathway [19]. Neovascularization was impaired in a model of hind limb ischemia in BKR2 knockout animals as compared to wild type controls [19]. In consequence vessel density and blood flow were impaired [19]. Other studies have shown that Ang II can mediate renal production of BK, nitric oxide (NO) and cGMP via AT2 receptor [9, 10, 20, 21]. Similarly, Ang II stimulates cGMP in stroke-prone hypertensive rats by stimulating BK via the AT2 receptor [9]. A recent study suggests that AT2 receptors participate in BK-dependent vasodilation induced by Ang II [22]. Vasodilation of isolated rat arteries was maximally inhibited when both BKR2 and AT2 receptor were blocked [22].

Interestingly, vasodilation is a prerequisite for initiation of angiogenesis [23]. It is therefore interesting that a vasoconstrictor molecule such as Ang II mediates its effect via one of its receptors, i.e. AT2 receptor, through specific pathways, upregulated in response to hypoxia, via a vasodilator molecule receptor, i.e. BKR2. Still, the precise nature of the AT2-BK interaction has not been clarified to date. In endothelial cells, Ang II reduces intracellular pH levels and this may activate acid-optimum kinogenases that would cleave stored kininogens to produce BK and in turn NO [10, 11]. We therefore speculate, that BK-induced NO production via BKR2 may play a role in AT2 mediated angiogenesis. Further characterization of the intracellular pathways upregulated in response to hypoxia are needed to fully understand the roles of hypoxia and the Ang II-BK interaction. The cells responsible for the observed effects remain undefined since our system is a mixture of cell types, further experiments using different cell types should be performed to answer more specific questions since our method is not suitable for this. Our model dealt with healthy adult mouse hearts *in vitro*, the role of Ang II on angiogenesis *in vivo* has not been studied nor have we used hearts of diseased animals. These will be the topic of future studies.

In conclusion, Ang II induces angiogenesis *in vitro* in the healthy, adult, mouse heart under hypoxia. The mechanism behind Ang II-induced angiogenesis requires the AT2 receptor as well as an interaction with the BKR2. Understanding the role and interaction of these receptors in pathophysiological situations such as hypertension and left ventricular hypertrophy, *in vivo* might lead to new insights for possible treatments.

Materials and Methods

Animals

Experiments were performed with hearts of different mouse strains: Black six wild type mice, AT1 KO mice (The Jackson Laboratory, Maine USA: B6.129P2-Agtr1^{tm}1Unc) and BK2 KO mice (The Jackson Laboratory, Maine USA: B6;129S7-Bdkrb2t^{m}1Jfh). AT2 KO mice were obtained from Dr L. Hein as previously described [24] All hearts were obtained post mortem. The animals were euthanized and the hearts immediately transferred to PBS. Within half an hour after death small pieces (1mm^3) of the mouse myocardium (left ventricle) were embedded in fibrin gel. All experiments conformed with the rules of the Swiss Federal Act on Animal Protection (1998) and were approved by the Veterinary Department of the Kanton of Basel Stadt(Switzerland). Age of mice ranged from 12 to 14 weeks.

Assay of angiogenesis *in vitro*

The assay utilized was a three-dimensional angiogenesis *in vitro* assay of the heart established in our laboratory [14]. Briefly, gels were made by preparing a fibrin gel solution: 3 mg of Fibrinogen (Sigma-Aldrich, Buchs, Switzerland) per ml serum-free DMEM (Oxoid, Basel, Switzerland) with 0.1U/ml of thrombin (Sigma-Aldrich, Buchs, Switzerland) on ice. 100 µl/ well of this fibrin gel solution were immediately pipetted into each well of 48-well plates and allowed to polymerize for 45 min at 37°C. Gels were overlaid with 500 ml serum-free DMEM for at least 30 minutes. The medium overlaying the gel was removed and 0.5 - 1mm3 cubes from the left ventricular myocardium of the heart were placed onto the gels in each well and overlaid with 100 ml of fibrin gel solution. After 45 minutes of polymerization, gels were overlaid with 500 µl standard DMEM containing the indicated concentration of fetal calf serum (FCS, Oxoid, Basel, Switzerland). Heart explants were then exposed to stimulants and or inhibitors and incubated under normoxia (21% O_2) and hypoxia (3% O_2) conditions for 7 days, with addition of stimulants/inhibitors every second day: hr VEGF164 (R&D systems, Minneapolis, MN); HOE140 (D-Arg-[Hyp3, thi5,D-tic,Oic8]-BK) (Sigma-Aldrich AG); Angiotensin II Human Acetate (Sigma-Aldrich AG); Losartan Potassium 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5-ylphenyl)benzyl] imidazole-5-methanol-monopotassium salt (Merck Sharp & Dohme-

Chibret AG, Glattbrugg, Switzerland), CCP42112 Nicotinoyl-Tyr-Lys(Z-Arg)-His-Pro-Ile-OH (Bachem AG, Budendorf, Switzerland), PD123,319 Ditrifluoroacetate (Fluka Chemie GmbH, Buchs, Switzerland).

Inhibitors were added freshly 20 minutes before stimulants and included throughout the incubations where neceassry. Fibrin gels were protected from degradation by adding 300 µg/ml e-amino-caproic acid (Sigma-Aldrich) every third day. After 7 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). The extent of sprout formation was determined for each condition in octuplicates by comparison with a standardized scale (angiogenic index) by two independent investigators , one of which was blinded to the exact experimental conditions tested, and averaged. In addition the angiogenic index was defined, in selected experiments, with the help of an image analysis software (AnalySIS Pro, Soft Imaging System, Gloor Instruments, Uster, Switzerland). The index is defined by the relation between the total area of outgrowing cells to the area of the embedded heart tissue. Based on this a representative scale from 0 to 4 was defined.

Characterization of cells & tissue

For characterization of outgrowing cells and sprouts specific cell markers were directly applied to heart cultures in the gels: The contents of the well were fixed over night with 4% Paraformaldehyde (Merck AG, Dietikon, Switzerland), washed with PBS and incubated for 3 hours with specific cell markers: Fluorescein conjugated GSL I – IB_4 (20 □g/ml; Rectolab S.A., Servion, Switzerland) for endothelial cells, Cy3-conjugated anti-□-smooth muscle actin (1:100; SMA; Fluka Chemie GmbH, Buchs, Switzerland) for smooth muscle cells or pericytes and Hoechst dye (Polysciences Europe GmbH, Eppelheim, Germany) for visualization of cell nuclei. After incubation with cell markers the probes were extensively washed with PBS.

Statistical analysis

All results depicted represent experiments repeated on at least five separate occasions and using at least five different heart explants. Each single condition was performed in octuplicate wells. Data points represent the mean of at least five single experiments ± SEM. Statistical analysis was performed where necessary with SPSS for Mac OS X (SPSS, Inc., Chicago, USA). Statistical significance ($p < 0.05$) was established by using non-parametric analysis; Kruskal-Wallis and Mann-Whitney tests were performed accordingly.

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4. CONCLUSIONS AND KEY FINDINGS

The aim of this study was to investigate the role of Angiotensin II and Nitric Oxide on angiogenesis in the heart *in vitro*, independent of blood pressure using our newly developed system. We also aimed to understand the mechanisms involved in these responses. Both Ang II and NO are crucial in regulating blood pressure and involved in many cardiovascular pathologies.

Hypertension is a mayor risk factor for many diseases, untreated it can lead to arteriosclerosis, coronary heart disease, stroke, loss of vision and kidney failure, among others. Most population-based studies confirm that hypertension increases an individual's risk of cardiovascular consequences approximately two to three times [1].

Coronary Heart Disease is the leading cause of death worldwide. More than 60% of the global burden of coronary heart disease occurs in the western world. In middle-income countries cardiovascular disease is responsible for 18% of the disability-adjusted life years (DALYs) lost, or the 'healthy years of life lost'. [2].

While genetic factors play an important part, 80 to 90% of the people dying from coronary heart disease have one or more mayor risk factors that are influenced by lifestyle. The main risk factors associated with coronary heart disease are high blood pressure, tobacco use, high cholesterol, alcohol consumption and obesity.

Stroke is also a mayor killer, annually 15 million people worldwide suffer from a stroke, of these 5 million die and 5 million are left permanently disabled, placing a burden of family and communities. The mayor risk factors for stroke are similar to those of coronary heart disease, with high blood pressure and smoking being the most significant modifiable risks. Treating hypertension can reduce the risk of a stroke by up to 40% [2].

Table 1 summarizes some basic facts and figures from around the world concerning heart disease, stroke and the response of the local scientific community. The economic losses that occur due to these two conditions are enormous, both to families, businesses and communities, even without including the incalculable value of a human life. The scientific community has responded to this challenge with a huge amount of research being produced on these topics. In 2004 alone the number of

published clinical trials in the National Library of Medicine (Pub Med) for coronary heart disease is 57 507, an astonishing number when compared to the 6539 trials published in the same period for HIV/AIDS.

Country	Population *Thousands*	Heart Disease		Stroke		Smoking Prevalence	Research 1991-2001
		DALYs lost per 1000	Mortality	DALYs lost per 1000	Mortality		
Argentina	37981	6	34292	6	22668	25%	110
South Africa	44759	9	27013	11	30306	29%	77
Switzerland	7171	4	10746	2	4508	33%	440
USA	291038	8	514450	4	163768	25%	12502

Table 1: World Data Table obtained form the Atlas of Heart Disease ad Stroke published by the WHO [2].

Given the importance of understanding the heart and hypertension and the importance of improving blood circulation, our work has focused on the topic of vascular biology and angiogenesis.

4.1 Hypertension and Angiogenesis

In the review 'Hypertension and Angiogenesis' published in 2003 in the Current Pharmaceutical Design journal we attempted to discuss some basic mechanisms of angiogenesis and their involvement in hypertension.

We also describe, the proposed pathways and actions of the main actors in the RAAS suggesting that accumulation of BK in response to ACE inhibition may be responsible for the resulting angiogenesis.

As a final conclusion we comment on the existence of evidence of abnormal angiogenesis in cardiovascular disease and increasing evidence that it has a role in the pathogenesis of hypertension. Since many of the available antihypertensive agents have beneficial effects on angiogenesis they may improve the condition of microvascular rarefaction and thus prevent organ damage.

4.2 A versatile *in vitro* assay for investigating angiogenesis of the heart

In 2004 we published a paper in Experimental Cell Research entitled 'A versatile *in vitro* assay for investigating angiogenesis of the heart'. In this paper we describe a new method for investigating angiogenesis [3].

At the time most experiments were being performed *in vivo* since no appropriate *in vitro* model was available. *In vivo* experiments require a large number of animals, are difficult to perform and are often associated with pain to the animals. A new *in vitro* model would be desirable to solve some of these problems. Therefore, we aimed to investigate, set up and validate a model of *in vitro* angiogenesis of the heart and to then use this model to asses the angiogenic response of the heart to known angiogenic stimuli.

In our study [3] we found that both hypoxia and serum (5%) are required for angiogenesis to occur in the adult mouse heart *in vitro*. During hypoxia angiogenesis occurred already at basal levels without addition of growth factors. Addition of growth factors induced substantial levels of angiogenesis in hypoxia. We histologically analyzed the heart pieces after 10 days of hypoxic culture and found a fairly well preserved architecture of the cardiac tissue and no necrosis. To investigate the possibility of using this method to test inhibitors of angiogenesis we used rapamycin. Inhibition was easily measured; this new model appears appropriate for testing large numbers of compounds to find out if they might be pro-or anti-angiogenic in the heart. To further describe this assay we analyzed the morphology of the different sprouts. Not every substance tested induced the same type of branching. However, sprouts were always composed by endothelial cells. Smooth muscle cells or pericytes aligned along the sprouts, which is consistent with a stage of early vessel maturation. A lumen was not present, also implying that this model may correspond to an early stage of angiogenesis. Angiogenic potential is reduced with age and we found the same to be true in our model. The older the mice, the less angiogenesis was observed in the heart explants prepared from it. Our system was also tested with a piece of human heart and results were similar to those obtained with rodent hearts.

We conclude that angiogenesis of the heart *in vitro* can be investigated with a simple assay that allows a large series of experiments to be carried out in a relatively short time and with a minimum amount of animals. We demonstrated the reliability of our model by reproducing known *in vivo* and *in vitro* phenomena and we have seen that

our model is suitable to investigate the actions of different substances on angiogenesis of the heart. An important finding was that the model is suitable for studying both substances that induce angiogenesis as well as substances that inhibit it.

4.3 iNOS is required for *in vitro* angiogenesis of hypoxic healthy mouse hearts

We have recently submitted a paper entitled 'iNOS is required for *in vitro* angiogenesis of hypoxic healthy mouse hearts'.
In this brief report we have investigated the role of iNOS on angiogenesis of the mouse heart and aortae under hypoxia. We first used pharmacological inhibitors, either general NOS inhibitors or specific iNOS inhibitors. We found that the heart is more sensitive to the different inhibitors than aortae. When we repeated experiments using iNOS knock out animals we obtained very clear results. We found that in aortae, growth was reduced when stimulating with various growth factors. However, *in vitro* angiogenesis of the hypoxic heart in iNOS knock out mice, was totally absent. None of the growth factors tested was able to induce any sprout formation.
We therefore conclude, that organ specific pathways must exist for angiogenesis; and that iNOS is essential for angiogenesis of the hypoxic mouse heart.

4.4 Angiotensin II induces angiogenesis *in vitro* through an AT2 – BKR2 pathway in the hypoxic mouse heart

The last of my research is described in 'Angiotensin II induces angiogenesis *in vitro* through an AT2 – BKR2 pathway in the hypoxic mouse heart' a paper we are about to submit pending approval by Merck Sharp and Dohme-Chibret AG.
In this paper we studied the role of Ang II on angiogenesis of the mouse heart in hypoxia. We aimed to unravel the role of the AT receptor subtypes in angiogenesis induced by Ang II and to study downstream effectors that might be involved in this response.
We first tested the angiogenic capacity of Ang II and found it to be angiogenic only during hypoxia. We then used various pharmacological tools to unravel the receptors involved. We found that the AT1 antagonist Loartan does not block sprouting but an AT2 antagonist does. An AT2 agonist also induced similar levels of sprouting as Ang II. The role of AT2 receptor in angiogenesis was corroborated by the use of knock out animals. In AT1 knock out mice Ang II induced sprouting and this was abrogated by

the use of an AT2 inhibitor. On the other hand, AT2 knock out mice did not induce an angiogenic response when stimulated with Ang II. This allowed us to conclude that AT2 receptor was the one responsible for angiogenesis in our mouse heart *in vitro* model. Thereafter, we decided to look at downstream effectors.

Based on the literature [4] we decided that BKR2 might be involved. We used both pharmacological tools as well as transgenic animals to study whether BK might be involved in this response. We found that sprouting induced by Ang II in wild type animals could be inhibited with a BKR2 antagonist. When we used the BKR2 knock out mice we were not able to induce any sprouting with Ang II.

These results fit in with a model of interaction between Ang II and BK proposed by Volpe et al that can be seen in figure 8.

We thus conclude that Ang II is angiogenic in an *in vitro* model of the adult mouse heart during hypoxia and that this response occurs via AT2 receptor and that BKR2 is also involved in this response.

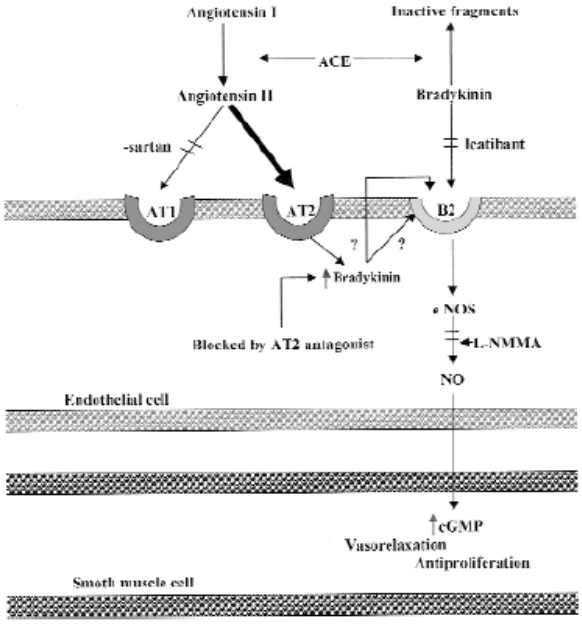

Figure 8: Proposed interactions among the AT2 receptors, the bradykinin and nitric oxide systems. ACE= angiotensin-converting-enzyme; cGMP= cyclic guanosin monophospahte; eNOS= endothelial nitric oxide synthase; L-NMMA= NG-monomethyl-L-arginine. Figure from M. Volpe and P de Paolis, 2000 [4]

4.5 Final Conclusion

Our work has ranged from the design of a new angiogenesis *in vitro* model to using it to study angiogenesis in healthy adult mouse hearts. Using this model we have studied the roles of Ang II and NO on angiogenesis of the healthy mouse hearts under hypoxia and achieved our objectives resulting in the following conclusions:

1 The *in vitro* model of the mouse heart is suitable for studying angiogenesis in adult mouse hearts under hypoxia and normoxia.

2. Sprouts induced in the mouse heart under hypoxia, are composed by endothelial cells with smooth muscle cells/pericytes aligned along the sprout.

3. PDGF, VEGF and FGF were able to induce sprouting in the adult mouse heart under hypoxia.

4. iNOS is essential for angiogenesis of the adult mouse heart under hypoxia

5. Ang II is angiogenic in the adult mouse heart under hypoxia

6. AT2 receptor is responsible for the sprouting induced by Ang II

7. BKR2 is involved in the angiogenic response due to the action of Ang II on its AT2 receptor.

4.6 References

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5. OUTLOOK

During the process of writing a thesis one learns a considerable amount and realizes all that could still be done and all the many questions that remain unanswered. I would like to outline some of these ideas.

1a.

We have described and characterized a novel *in vitro* model of heart angiogenesis. The model may have a lot of potential and could be used for various applications such as:

- Screening of compounds that might be angiogenic, that could later be used in therapeutic angiogenesis
- Screening of anti-angiogenic compounds that might be later used in cancer therapy
- Study diseased hearts as well as normal ones, for example hearts with Left Ventricular Hypertrophy, diabetes, etc.

1b.

Our assay also provides a good model to study cell interactions at the initiation of angiogenesis. It could be further utilized to:

- Study the cells that are involved at different stages of sprouting
- Study expression of receptors of interest

2.

In our brief report on the role of iNOS questions still remain unanswered such as:

- What is the role of eNOS in angiogenesis of the heart?
- Can angiogenesis be re-established by donation of NO?
- Can angiogenesis be re-established by induction of iNOS?

3.

In our paper on the role of Ang II on angiogenesis of the mouse heart in hypoxia also questions remain unanswered such as:

- What are the expression levels of AT1 and AT2 receptors in the mouse heart in the different conditions analyzed?
- What is the role of NO in the AT2 /BKR2 pathway?
- What signalling molecules are responsible for the angiogenic response in response to Ang II and to BK?

A lot of additional questions remain still unanswered. A lot of work is still to be done, but that's the magic of science, the work is never finished and there are always more things to discover and learn about. As Mahatma Gandhi said, "Live as if you were to die tomorrow. Learn as if you were to live forever"

6. ABBREVIATIONS

ACE	angiotensin converting enzyme
Ang II	angiotenisn II
BK	bradykinin
BKR1	bradykinin receptor 1
BKR2	bradykinin receptor 2
cGMP	3',5'-cyclic guanosine monophosphate
DAG	diacylglycerol
DALYs	disability-adjusted life years
EC	endothelial cells
eNOS	endothlial nitric oxide synthase
FGF	fibroblast growth factor
GPCR	G-protein-coupled receptors
GTP	guanosine 5'-triphosphate
HIF-1	hypoxia inducible factor 1
iNOS	inducible nitric oxide synthase
IP3	inositol triphosphate
KKS	kallikrein-kinin system
LVH	left ventricular hypertrophy
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthases
PLC	phospholipase C
PRCP	prolycarboxypeptidase
RAAS	renin angiotensin aldosterone system
SMC	smooth muscle cells
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells

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