THE ANGIOGENIC RESPONSE IN HYPOXIC HEART
Experimental studies in mice

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ABSTRACT

Coronary artery disease is the leading cause of death in the western world today. Although induction of angiogenesis would appear to be an ideal therapeutic strategy, clinical trials of pro-angiogenic factors have proved disappointing. Angiogenesis is a complex process involving many signaling pathways and mediators, and further insights into the underlying cellular and molecular mechanisms are urgently needed. Here, we used two mouse models, systemic hypoxia and myocardial infarction (MI), to study the effects of hypoxia on angiogenesis in the myocardium, and the cellular and molecular mechanisms involved.

Hypoxia-inducible factor-1α (HIF-1α) is an important transcriptional regulator of angiogenesis. Small ubiquitin-related modifier-1 (SUMO-1) has been shown to stabilize transcription factors and modulate their activity. In our mouse model of systemic hypoxia, we showed that SUMO-1 expression is enhanced by hypoxia in brain and heart. Furthermore, SUMO-1 co-localizes and directly interacts with HIF-1α under hypoxic conditions, indicating that hypoxia-mediated increases in SUMO-1 expression could modulate HIF-1α function.

We combined our mouse model of systemic hypoxia with a model of MI and showed that chronic hypoxia protects the heart from infarct injury and promotes angiogenesis. A proteomics analysis demonstrated that protein disulfide isomerase (PDI) is upregulated in the myocardial capillary endothelial cells of mice exposed to chronic hypoxia. Furthermore, PDI knockdown in endothelial cells *in vitro* increases apoptosis and inhibits migration and adhesion, indicating that PDI may play an integral role in angiogenesis.

Endoglin is a co-receptor for transforming growth factor-β. In our mouse model of MI, we showed increases in endoglin expression in endothelial cells in the heart one week after surgery. Similarly, endoglin expression is increased in endothelial cells *in vitro* after exposure to hypoxia. Furthermore, we showed that hypoxia promotes activation of the endoglin/ALK-1/SMAD1/5 but not the endoglin/ALK-5/SMAD3 signaling pathway in endothelial cells. The induction of this pathway represents another potential mechanism for regulation of angiogenic responses in endothelial cells after MI.

The results presented advance our understanding of the complex pathways involved in hypoxia-mediated angiogenesis in the heart. Our findings could play a role in identifying new strategies for the treatment of ischemic heart disease.

LIST OF PUBLICATIONS

The thesis is based upon the following papers, referred to in the text by their roman numerals:

Paper I **Increase of SUMO-1 expression in response to hypoxia: direct interaction with HIF-1α in adult mouse brain and heart *in vivo***

Ruijin Shao, Fu-Ping Zhang, Fei Tian, Anders Friberg, Xiaoyang Wang, Helen Sjöland, Håkan Billig

FEBS Letters 2004, 569:293-300

Paper II **Expression of protein disulfide isomerase is increased in vascular endothelial cells during myocardial infarction in mice exposed to chronic hypoxia: role in angiogenesis?**

Fei Tian, Johannes Wikström, Helen Karlsson, Xianghua Zhou, Helén Sjöland, Li-Ming Gan, Levent M. Akyürek, Jan Borén

In revision 2008

Paper III **Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling *in vivo* and *in vitro***

Fei Tian, Xianghua Zhou, Erik Larsson, Carl-Henrik Heldin, Jan Borén, Levent M. Akyürek

In revision 2008

LIST OF ABBREVIATIONS

ALK	Activin receptor-like kinase
bcl-X	B-cell lymphoma-like X
BMP	Bone morphogenetic protein
BRE	BMP-responsive element
CAGA	SMAD3-responsive element
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
HAEC	Human aortic endothelial cells
HIF-1	Hypoxia-inducible factor-1
HRE	Hypoxia-responsive element
HUVEC	Human umbilical vascular endothelial cells
Id1	Inhibitor of differentiation-1
LAD	Left anterior descending coronary artery
MI	Myocardial infarction
mRNA	Messenger RNA
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PECAM-1	Platelet endothelial cell adhesion molecule-1
PFA	Paraformaldehyde
siRNA	Small interfering RNA
SUMO-1	Small ubiquitin-related modifier-1
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

1.1. Clinical background

It has been known for many years that the incidence of myocardial infarction (MI) is lower in people living at high altitudes. Hurtado first reported this clinical benefit from a study of individuals living 4000 m above sea level in Peru (1), and an epidemiological study from New Mexico showed that even moderate elevations (2100 m) could result in protection against death from ischemic heart disease (2). The protective effects of high-altitude hypoxia have been confirmed experimentally (3-5), and knowledge of the underlying cellular and molecular mechanisms could identify potential therapeutic targets for the treatment of cardiovascular disease.

1.2. Myocardial hypoxia

Myocardial hypoxia is a state of reduced oxygen supply to the heart. The most common causes are systemic hypoxia, characterized by a drop in O_2 saturation in the arterial blood but adequate perfusion (6), and ischemic hypoxia, induced by the reduction or interruption of coronary blood flow.

Systemic hypoxia can be physiological, as observed in populations living at high altitudes (see *Clinical background*). It is also observed in mountaineers and in individuals suffering from chronic cor pulmonale, cyanotic heart disease and chronic obstructive lung disease.

Ischemic hypoxia occurs in individuals suffering from ischemic heart disease and, acutely, MI. The effects are usually more severe than with systemic hypoxia as, in addition to the reduced O_2 supply, there is also a substantial reduction in the clearance of metabolites. In contrast to systemic hypoxia, which affects the whole myocardium, the effects of ischemic hypoxia are limited to the area supplied by the affected coronary artery.

1.3. Definition of angiogenesis

Angiogenesis is the creation of new blood vessels, predominantly capillaries (7), from pre-existing blood vessels. The term was introduced in 1935 by Hertig to describe the formation of new blood vessels in the placenta, and Folkman later described angiogenesis as the neovascularization accompanying the growth of solid tumors (8).

Angiogenesis can be both physiological, as observed in fetal and childhood growth, formation of the corpus luteum and in wound healing and pathological, as induced by tumors, MI, stroke, chronic inflammation, psoriasis and diabetic retinopathy (9-12).

1.4. Angiogenesis in systemic hypoxia

There is considerable evidence to show that systemic hypoxia promotes angiogenesis in the brain (13, 14), retina (15) and lung (16). However, the effect of systemic hypoxia is not universal. For example, it does not result in new vessel formation in skeletal muscle (17). Furthermore, there are conflicting results regarding the development of myocardial capillaries in animals exposed to hypoxia. Canepa et al. (18) and Smith

and Clark (19) found a decrease in the capillary density in chronically hypoxic guinea pigs and rats. In contrast, Miller and Hale (20) and Zhong et al. (21) found increased capillary density in chronically hypoxic rats. Thus, it has not been fully elucidated whether and to what degree systemic hypoxia induces myocardial angiogenesis.

1.5. Angiogenesis in the infarcted heart

It is well-established that hypoxia-induced angiogenesis is important to promote healing in the infarcted heart. New vessels begin to appear in the infarcted area three to four days after infarction (22-24), and the infarcted area is rich in capillaries one week post MI (25, 26). The newly formed vessels allow increased blood flow, thus increasing the amount of oxygen delivered to affected tissue to salvage ischemic heart tissue (27). After four to eight weeks, the new vessels undergo a maturation process leading to the formation of pericyte-coated vessels and regression of many capillaries (24, 25, 28).

In addition, preexisting arteriolar connections can be recruited to bypass the site of occlusion following chronic or acute occlusion of a major artery (29, 30). This process is termed arteriogenesis and is achieved by the rapid proliferation of preexisting collateral arteries and by the maturation of capillaries into mature arterioles. These native collaterals, which are not used to enhance perfusion under normal conditions, can thereby dramatically increase the lumen to provide enhanced perfusion to the jeopardized ischemic regions.

1.6. Process of angiogenesis

Angiogenesis consists of an activation phase and a resolution phase (30) (Figure 1). Pro-angiogenic factors such as vascular endothelial growth factor (VEGF) are released in response to tissue injury or hypoxia and activate endothelial cells (31, 32). Endothelial cells loosen their contacts with their basement membrane and their supporting peri-endothelial cells (pericytes in small vessels and smooth muscle cells in large vessels), leading to increased vascular permeability and deposition of fibrin into the extravascular space, vessel wall disassembly and degradation of the basement membrane. The activated endothelial cells migrate on and into the fibrin scaffold and invade the underlying extracellular matrix towards the angiogenic stimulus and proliferate.

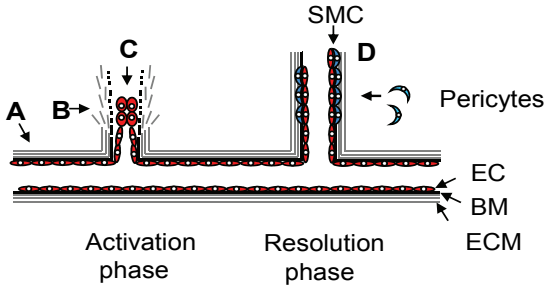

Figure 1. *Process of angiogenesis. **(A)** An angiogenic stimulus activates endothelial cells (EC), leading to **(B)** degradation of the extracellular matrix (ECM) and basement membrane (BM), and **(C)** proliferation, adhesion and migration of EC. **(D)** In the resolution phase, EC are released, and smooth muscle cells (SMC) and pericytes are recruited.*

Ultimately, they align to form the capillary lumen. Once a new vessel

has been formed, angiogenesis enters the resolution phase. Proliferation and migration of endothelial cells are inhibited and a new basement membrane is secreted. The junctional complexes between the endothelial cells as well as with the basement membrane mature and peri-endothelial cells are recruited and differentiate.

1.7. Regulation of angiogenesis

Angiogenesis is mediated and regulated by a wide array of angiogenic inducers, including growth factors, chemokines, enzymes, endothelial cell-specific receptors, and adhesion molecules (30). Hypoxia is known to stimulate the release of various pro-angiogenic factors, including platelet-derived growth factor, fibroblast growth factor (FGF) 1 and 2 (33), and transforming growth factor-β (TGF-β) (34). In addition, hypoxia upregulates the expression of VEGF and its receptors, partly through activation of hypoxia-inducible factor-1α (HIF-1α) (35).

Here, we briefly introduce the hypoxia-induced pro-angiogenic molecules studied in this thesis, namely HIF-1α, small ubiquitin-related modifier 1 (SUMO-1), protein disulfide isomerase (PDI) and the TGF-β receptor endoglin.

1.7.1. HIF-1α

HIF-1 is a heterodimeric transcription factor consisting of two subunits: HIF-1α and HIF-1β (36, 37). Both subunits belong to the basic helix-loop-helix PER-ARNT-SIM family of transcription factors (38), which induce gene expression by binding to the hypoxia-responsive element (HRE) (39, 40). In the presence of O_2, HIF-1α subunits are modified by ubiquitin and thereby directed to proteosomal degradation (41-44). By contrast, under hypoxic conditions, HIF-1α is stabilized, translocated to the nucleus, and complexed with HIF-1β to promote HRE-driven transcription of O_2-regulated genes (45), including erythropoietin, glycolytic enzymes, inducible nitric oxide synthase, heme oxygenase-1 and VEGF (34, 46).

1.7.2. SUMO-1

SUMO is a member of the ubiquitin-like protein family (47-50). It is a relatively small protein of around 100 amino acids. Although it shares only 18% sequence identity with ubiquitin, it has substantial 3D structural similarity (51, 52). There are three confirmed isoforms: SUMO-1, SUMO-2 and SUMO-3.

Post-transcriptional modification of proteins by SUMO (termed sumoylation) differs from ubiquitination as sumoylated proteins are not targeted for degradation. Instead, sumoylation appears to modulate protein properties such as subcellular localization, protein-protein interactions, protein stability, and transcription activities (53-55). Studies have shown that SUMO-1 interacts with various transcription factors and modulates their activity (56, 57). The β subunit of HIF-1 has been reported to be conjugated to SUMO and to influence its transcriptional activity (58). However, an association between

SUMO-1 and HIF-1α has not previously been reported.

1.7.3. PDI

PDI, the first characterized protein disulfide isomerase (59), is a member of the thioredoxin superfamily. It is a highly abundant endoplasmic reticulum (ER) luminal protein in mammalian cells, and constitutes about 0.8% of total cellular protein (60). To date, 17 putative protein disulfide isomerases have been identified in human (61).

PDI plays a role in protein folding by catalyzing the formation of native disulfide bonds and disulfide bond rearrangement. In addition, PDI is essential for cell survival, and upregulation of PDI results in resistance to apoptosis after hypoxia in astrocytes (62) and ischemic brain and heart (62, 63).

PDI has also been demonstrated on the surface of numerous cell types, including platelets, lymphocytes, hepatocytes, fibroblasts and endothelial cells (64-68), and hypoxia has been reported to increase PDI expression in endothelial cells (69). However, a role for PDI in angiogenesis has not yet been established.

1.7.4. Endoglin

TGF-β plays an important role in angiogenesis by binding to specific serine/threonine kinase receptors (70, 71). The accessory TGF-β receptor endoglin is a homodimeric transmembrane glycoprotein (72). It is predominantly expressed by vascular endothelial cells where it regulates endothelial cell proliferation and migration, processes crucial for angiogenesis (73). Endoglin forms complexes with two different TGF-β type I receptors expressed by endothelial cells, activin receptor-like kinase-1 (ALK-1) and ALK-5, to promote angiognesis by regulating TGF-β/ALK signaling (74) (Figure 2). ALK-1 activation induces phosphorylation of the transcription factor SMAD1/5 and has been proposed to stimulate endothelial cell proliferation and migration (75), whereas ALK-5 activation phosphorylates SMAD2/3, which has been shown to inhibit these processes (76). Recent reports indicate that hypoxia increases expression of endoglin in endothelial cells (77, 78), and in infarcted mouse hearts (79). However, the preferentially activated signaling pathway downstream of the hypoxia-induced increase in endoglin expression has not yet been studied.

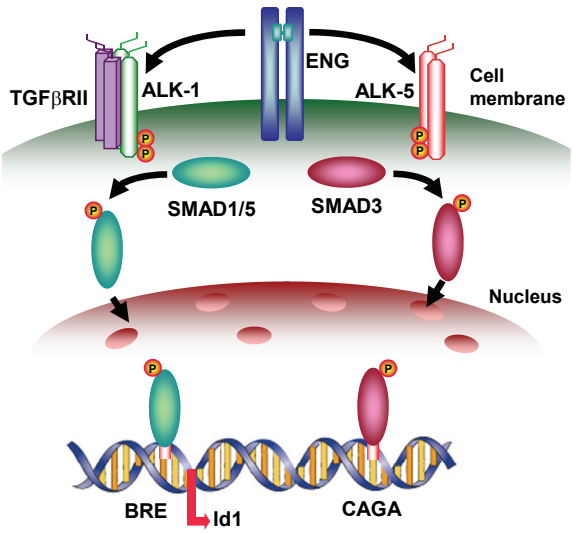

Figure 2. *Endoglin (ENG) signaling is mediated through two separate pathways.*

2. AIM OF STUDY

The overall aim of this thesis was to study the effects of hypoxia on angiogenesis in the myocardium, and the cellular and molecular mechanisms involved.

The specific aims of this thesis were:

1. To investigate the regulation of SUMO-1 expression and the interaction between SUMO-1 and HIF-1α in mouse brain and heart in response to chronic systemic hypoxia (**Paper I**).

2. To study the effect of hypoxia-induced angiogenesis on myocardial injury in the infarcted mouse heart and the role of PDI in hypoxic endothelial cells (**Paper II**).

3. To identify which endoglin signaling pathway is activated in the infarcted mouse heart and hypoxic endothelial cells (**Paper III**).

3. METHODS AND METHODOLOGICAL CONSIDERATIONS

3.1. In vivo *studies*

3.1.1. Animals

C57BL/6 mice aged 8-10 weeks (M&B, Ejby, Denmark) were used in all the experiments. Animal experiments were approved by the animal ethics committee in Gothenburg.

3.1.2. Mouse model of systemic hypoxia

Systemic hypoxia produces long-term effects that could influence the vascular structure and function, predominantly in the brain (80-82). Many experimental studies have been performed to evaluate the effects of hypoxia on the cardiovascular system, but predominantly investigated exposure to acute hypoxia (83, 84). Here we used a model of chronic systemic hypoxia to study the effects of long-term hypoxia on heart function.

Chronic hypoxia has been investigated in dogs and rodents (4, 85-87). Mice are more frequently used in experimental studies as they are cheap, easy to handle, and suitable for human comparisons.

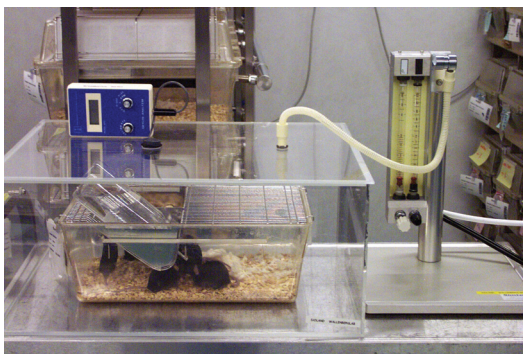

Figure 3. *Mice in a hypoxic (10% O_2) chamber.*

In this thesis, mice were placed in a specially designed hypoxic chamber (Figure 3) and exposed to a low oxygen level for four days (Paper I) or one or three weeks (Paper II). The oxygen concentration was continuously monitored with an oxygen sensor and precisely maintained at 10%. Control mice were kept in room air.

Use of this chamber avoided even short periods of unwanted exposure to room air, e.g., during feeding and maintenance. This is an important consideration as it has been demonstrated that exposure of animals to room air for only an hour a day substantially reduces the myocardial effects of exposure to hypoxia for the remaining time (88). In addition, we chose to use 10% O_2 because a major focus of our study was to examine protein expression. Hypoxic stress is known to inhibit protein synthesis (89), but 10% O_2 has previously been shown not to inhibit protein synthesis (90-93).

3.1.3. Mouse model of MI

Preclinical models of myocardial ischemia have been reported in several large animal species, including pigs (94, 95), dogs (94, 96, 97) and goats (98). The model that most closely resembles the response seen in humans is the pig ameroid model, which has been used in a variety of angiogenesis studies (95, 99). However, the expense and practical demands of porcine surgical facilities severely limit the extent of such studies (99-101).

A rodent model of acute MI was first developed in the rat (102). The major advantage of the rat over the mouse for surgery is that it is ten times larger. However, the advent of sophisticated microdissecting microscopes and microsurgical instruments has made mouse MI surgery as feasible as in the rat (103). In the past decade, animal surgery in cardiovascular research has now largely shifted from the rat to the mouse, and C57BL/6 is the most common mouse strain used (104).

Two models in mice have been developed by ligation of the left anterior descending coronary artery (LAD): the ischemia-reperfusion model (103) and the nonreperfused MI model (105, 106) The ischemia-reperfusion model is widely used to investigate genes involved in reperfusion injury (107), and the nonreperfusion model has been mainly used to study the genes involved in wound healing processes (108). The nonreperfusion model has also often been applied to the study of angiogenesis (26, 109) as it is known that infarct healing is associated with an angiogenesis response leading to formation of neovessels in the infarcted territory. Therefore, this model has proved useful in studies of pathophysiologic interactions of growth factors in the angiogenic response to MI, and is thus the model that we have used in this thesis.

In Papers II and III, MI was induced by permanent ligation of the left coronary artery (105). Mice were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg) and ventilated with room air. A left thoracotomy was performed in the third intercostal space. A suture (7-0 prolene) was tied around the main left coronary artery. The lung was re-expanded, the thoracotomy and skin incision were closed, and the animal was allowed to recover at 30°C. Age-matched control mice were subjected to the same surgery with the exception of coronary artery ligation (defined as sham surgery).

3.1.4. Echocardiography

Transthoracic color Doppler echo-cardiography was performed on isoflurane-anesthetized mice in Paper II, as previously described (110). Ultrasound scanning was performed using a high-frequency 15-MHz linear transducer (Sonos 5500, Agilent, Andover, MA) that was connected to an ultrasound system (HDI 5000, ATL Ultrasound, Bothell, WA) with a maximum frame rate of 230 frames/s. Ejection fraction was calculated using previously validated formulae (111). Coronary flow velocity was recorded in the first diagonal branch of the ligated LAD before and after infusion of adenosine (140 μg/kg/min i.v.).

3.1.5. Tissue preparation

Mice were killed with an overdose of pentobarbital at the end of experimental procedures. For immunohistochemistry and morphometry, tissues were fixed with a zinc fixative or 4% para-formaldehyde (PFA). Fixed organs were paraffin embedded and sectioned to 5 μm thickness (Papers I and III). For confocal microscopy (Paper II), the tissues were fixed in 4% PFA overnight, and sectioned to 100 μm thickness by vibratome. For immunoblotting and proteomic

studies, the left ventricles were separated, weighed, rapidly frozen in liquid nitrogen and stored at -80°C until assayed

3.1.6. Preparation of whole cell, cytosolic and nuclear protein extracts

The preparation of whole cell protein lysates was performed as described (112). Cytosolic and nuclear extracts were obtained by following a previously described protocol (113) with minor modifications (see Paper I). Protein concentrations were determined by BCA protein assay.

3.1.7. Immunoblotting and northern blotting

Immunoblotting was performed as previously described (114) to measure protein expression of HIF-1α, SUMO-1, PDI and Ang-1 in hypoxic hearts (Papers I and II) and endoglin, ALK-1, ALK-5, phosphorylated SMAD1/5 and phosphorylated SMAD3 in infarcted hearts (Paper III). Northern blotting was performed as described (115) to measure RNA expression of SUMO-1 in hypoxic hearts (Paper I).

3.1.8. Immunostaining

Paraffin sections were processed as described (116) to determine cellular localization of HIF-1α and SUMO-1 (Paper I), PDI (Paper II), endoglin, ALK-1 and phosphorylated SMAD1/5 (Paper III). To visualize capillary structures, immuno-fluorescence was used as described in Paper II.

3.1.9. Co-immunoprecipitation

Co-immunoprecipitation between HIF-1α and SUMO-1 in hypoxic hearts (Paper I) was investigated according to a previously described procedure (114).

3.1.10. Quantification of MI

Measurements of infarcted left ventricular areas in Paper II were carried out by light microscopy coupled with a computerized imaging system (Axiovision 3.0, Carl Zeiss, Jena, Germany). Three tissue sections (5 μm thick) were taken from the apex to the base of the left ventricle and stained with Masson's trichrome (117), staining viable areas red and necrotic tissues green.

As infarcts shrink over time, the infarcted wall will just be a thin layer of scar tissue after three weeks of ligation (106). To take into account the thinning of the infarcted myocardium, we applied the most widely used method for measuring infarcted size. Thus, we measured the epicardial and endocardial circumference of the infarction for each section, and divided this value by the total epicardial and endocardial circumference (117-120). Infarction size was then expressed as the percentage of total left ventricular circumference.

3.1.11. Analysis of capillary and arteriolar density

Capillaries were stained using platelet endothelial cell adhesion molecule 1 (PECAM-1) and arterioles were stained with smooth muscle α-actin (Paper II). Capillaries (approximately <10 μm diameter) were counted in five random computer captured

frames from left ventricular cross-sections. All arterioles (approximately <50 μm/diameter and ≤1 layer of smooth muscle cells) were counted in left ventricular cross-sections (26, 32, 121).

3.1.12. Proteomics analysis

Proteomic analysis in hypoxic hearts (Paper II) was performed as previously described (122).

3.2. In vitro *studies*

3.2.1. Cells

Human umbilical vascular endothelial cells (HUVEC) were cultured in EGM-2 medium (Paper II) and human aortic endothelial cells (HAEC) were cultured in Medium 200 (Paper III). Cells between passages three and six were incubated in either 21% O_2 (normoxia) or 1% O_2 (hypoxia). We used 1% O_2 as apoptosis does not occur with O_2 levels above 0.5% (123). Cells were washed twice with cold PBS before lysis in RIPA buffer supplemented with protease inhibitor. Cell debris was removed by centrifugation at 12,000 rpm for 20 min. Cells were exposed to normoxia or hypoxia for 24 h unless otherwise stated.

3.2.2. Immunoblotting

To measure the effect of hypoxia on expression of PDI in HUVEC (Paper II) and endoglin and ALK-1 in HAEC (Paper III), immunoblotting was performed as previously described (114).

3.2.3. Quantification of apoptosis, migration and adhesion

To determine the effect of PDI silencing on apoptosis, HUVEC were transfected with either negative control siRNA or PDI siRNA and incubated at normoxia or hypoxia for 48 h. Apoptosis was quantified according to the manufacturer's protocols (Paper II).

To determine the effect of PDI silencing on migration, HUVEC were transfected with either negative control siRNA or PDI siRNA, seeded in a transwell Boyden chamber and incubated at normoxia or hypoxia for 4 h. Cells that migrated to the lower face of the transwell membrane were stained and counted (Paper II).

To determine the effect of PDI silencing on adhesion, HUVEC were transfected with either negative control siRNA or PDI siRNA, seeded in 96-well plates (Chemicon) coated with collagen I, collagen II, collagen IV, fibronectin, laminin, tenascin, or vitronectin and incubated at normoxia or hypoxia for 90 min. Adhesion was quantified according to the manufacturer's protocols (Chemicon ECM cell adhesion kit) (Paper II).

3.2.4. Luciferase assay

To measure the effect of hypoxia on the activity of the BMP-responsive element (BRE) and the SMAD3-responsive element CAGA in HAEC (Paper III), cells were co-transfected with plasmid vectors encoding the luciferase reporter gene under the control of BRE, CAGA, constitutively active ALK-1 and dominant negative ALK-1. Transfection of HAEC was carried

out using electroporation by nucleofector. Luciferase activity was determined using a luminometer.

3.2.5. Real-time quantitative PCR

To measure the effect of overexpression of endoglin and ALK-1 on expression of *bcl-X* and *Id1* mRNA in hypoxic HAEC (Paper III), real-time quantitative PCR was performed according to a standard procedure. The following primers were used: human *bcl-X*, 5'-ACATCCCAGCTCCACATCAC-3' and 5'-TGCTGCATTGTTCCCAT-AGA-3'; human *Id1*, 5'-CTCCAGCACGTCATCGACTA-3' and 5'-CGCTTCAGCGACACAA-GAT-3'. A TaqMan assay for human 18S rRNA (Applied Biosystems) was used to control quantifications. Each reaction was performed in triplicates and the standard curve method was used for relative quantification of gene expression.

3.2.6. Proliferation assay

To measure the effect of overexpression of endoglin and ALK-1 on the proliferation of HAEC (Paper III), cells were transfected with plasmid vectors encoding endoglin and/or ALK-1QD or ALK-1KR. Cells were counted using a cell counter (Beckman Coulter, Miami, FL).

3.3. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Differences between the groups were tested for statistical significance by one-way or two-way analysis of variance (ANOVA). A P value of < 0.05 was considered statistically significant.

4. SUMMARY OF RESULTS

4.1. Paper I

The aim of this study was to investigate the regulation of SUMO-1 expression and the interaction between SUMO-1 and HIF-1α in mouse brain and heart in response to chronic systemic hypoxia.

4.1.1. Hypoxia increases expression of SUMO-1 and HIF-1α in mouse brain and heart

To allow the effects of systemic hypoxia to be investigated, mice were placed in a hypoxic chamber for four days. Immunoblotting and northern blotting showed that expression of both SUMO-1 protein and mRNA was significantly increased in the brain and heart from both female and male mice after hypoxic exposure compared with normoxic controls. Immunohistochemical staining revealed that the number of SUMO-1-positive cells was also significantly increased in brain and heart after hypoxic exposure. Immunoblotting analysis of nuclear and cytosolic extracts from brain and heart tissues showed that HIF-1α expression was present in the nuclear fraction from mice exposed to systemic hypoxia but not in extracts from normoxic controls.

4.1.2. SUMO-1 and HIF-1α co-localize and interact in hypoxic mouse brain and heart

SUMO-1 has previously been shown to interact with various transcription factors, including HIF-1β. Because the expression patterns for SUMO-1 and HIF-1α were similar in brain and

heart under hypoxic conditions, we investigated if these two molecules are co-expressed and if they interact. Double-labeled immunofluorescence analysis showed that both SUMO-1 and HIF-1α localized in the nucleus in neurons and cardiomyocytes in tissue sections from mice exposed to hypoxia, indicating that SUMO-1 and HIF-1α are co-expressed in the nuclei at hypoxia. Co-immunoprecipitation studies with anti-HIF-1α and anti-SUMO-1 antibodies showed that SUMO-1 conjugated with HIF-1α in brain and heart tissue from mice exposed to hypoxia.

4.1.3. Conclusion

We thus propose that the increased levels of SUMO-1 in mouse brain and heart observed after exposure to chronic systemic hypoxia could participate in the modulation of HIF-1α function. Following the publication of Paper I, it has been reported that HIF-1α is sumoylated by SUMO-1 in hypoxia conditions, leading to an increase in HIF-1α stability and transcriptional activity (124). These studies thus strengthen our proposal that hypoxia-mediated increases in SUMO-1 in mouse brain and heart could participate in the modulation of HIF-1α function.

4.2. Paper II

The aim of this study was to investigate the effect of hypoxia-induced angiogenesis on myocardial injury in the infarcted mouse heart and the role of PDI in hypoxic endothelial cells.

4.2.1. Chronic hypoxia improves survival and myocardial function in a MI mouse model

To investigate how previous exposure to chronic systemic hypoxia affects myocardial function after MI, mice were placed in a hypoxic chamber for one or three weeks before permanent ligation of the left coronary artery to induce MI. We observed that exposure of mice to chronic hypoxia for three weeks before MI resulted in improved survival and myocardial function and reduced infarction size three weeks after MI.

To further investigate this protection, we quantified the capillaries and arterioles by immunohistochemisty. Exposure of mice to chronic hypoxia for three weeks resulted in increased capillary density in the myocardium both before MI and three weeks after surgery. The arteriolar density was also higher in mice exposed to hypoxia three weeks after MI.

4.2.2. Hypoxia increases expression of PDI

To investigate the molecular mechanisms involved in this hypoxia-mediated angiogenesis, we performed proteomic studies to identify proteins that were regulated by chronic hypoxia in the myocardium. PDI was among one of the most upregulated proteins. Immunoblotting analysis confirmed increased expression of PDI in the myocardium of mice exposed to chronic hypoxia for three weeks. Staining of serial cross-sections indicated that capillary endothelial cells were the main cellular origin of PDI.

4.2.3. PDI knockdown increases apoptosis and inhibits migration and adhesion of endothelial cells

To study the functional consequences of PDI upregulation, we next investigated the role of PDI in HUVEC. Immunoblotting analysis showed increased PDI expression in HUVEC exposed to hypoxia for 24 h compared with normoxic controls. Transfection of HUVEC with PDI siRNA resulted in increased apoptosis and reduced migration and adhesion of HUVEC to all tested ligands in both normoxia and hypoxia. The PDI inhibitor bacitracin also inhibited the migration of HUVEC in normoxia and hypoxia.

4.2.4. Conclusion

The mouse model used in this study allows us to study the impact of chronic hypoxia on the infarcted heart and to reveal the underlying molecular mechanisms. Our results demonstrate that chronic hypoxia protects the heart from MI by promoting angiogenesis. Furthermore, we propose that hypoxia-induced upregulation of PDI in endothelial cells may play a role in this protection.

4.3. Paper III

The aim of this study was to identify which endoglin signaling pathway is activated by hypoxia in the infarcted mouse heart and hypoxic endothelial cells.

4.3.1. Hypoxia increases expression of endoglin, ALK-1 and SMAD1/5 *in vivo* and *in vitro*

To investigate the effect of hypoxia on endoglin expression *in vivo*, we induced MI in mice by permanent ligation of the left coronary artery, and killed the mice one or three weeks after surgery. Immunohistochemical analysis of left ventricle sections showed strong endoglin staining in peri-infarct areas and in the infarct core one week after MI. Co-localization studies indicated that endoglin was expressed in vascular endothelial cells. Immunoblotting analysis showed increased endoglin expression in left ventricle tissues from mice one week after MI compared with tissues from mice that were exposed to sham surgery.

We also used HAEC to investigate the effect of hypoxia on endoglin expression *in vitro*. Immunoblotting analysis showed increased endoglin expression in HAEC exposed to hypoxia for 24 h compared with normoxic controls.

We then investigated the effect of hypoxia on proteins downstream of endoglin both *in vivo* and *in vitro* to determine which endoglin signaling pathway (ALK-1/SMAD1/5 or ALK-5/SMAD3) is activated by hypoxia. Vascular endothelial cells from the infarcted myocardium expressed both ALK-1 and phosphorylated SMAD1/5. Immunoblotting analysis showed increased expression of both ALK-1 and phosphorylated SMAD1/5 in left ventricle myocardium one week after MI compared with tissues from mice that were exposed to sham surgery.

We also showed increased expression of ALK-1 in HAEC exposed to hypoxia for 24 h compared with normoxic controls. In contrast, we did not observe significantly increased expression of ALK-5 or phosphorylated SMAD3 in sections from the infarcted hearts.

4.3.2. Overexpression of endoglin and ALK-1 activates downstream genes and promotes endothelial cell proliferation

The SMAD1/5 complex modulates transcription by binding to specific BRE sequences, whereas SMAD3 binds to CAGA sequence motifs on the promoters of target genes. To further investigate the endoglin signaling pathway activated by hypoxia, we transfected HAEC with plasmid vectors encoding BRE or CAGA. Hypoxia increased BRE activity but did not affect CAGA activity. In addition, overexpression of endoglin and ALK-1 increased BRE but not CAGA activity.

Our bioinformatics analysis identified two genes (*Id1* and *bcl-X*) that contain BRE elements and are regulated by ALK-1/SMAD1/5 but not ALK-5/SMAD3 signaling. Real-time quantitative PCR showed that overexpression of both endoglin and ALK-1 increased mRNA expression of *Id1* and *bcl-X* mRNA in HAEC exposed to hypoxia for 24 h.

Overexpression of endoglin and ALK-1 consequently significantly increased the number of HAEC at both normoxia and hypoxia, but greater increases were seen at hypoxia.

4.3.3. Conclusion

Our results indicate that hypoxia promotes endothelial cell proliferation *in vivo* and *in vitro* by activating the endoglin/ALK-1/SMAD1/5 but not the endoglin/ALK-5/SMAD3 signaling pathway.

5. DISCUSSION

5.1. Why do we need further treatments for patients with MI?

Despite significant advances in myocardial revascularization techniques, coronary artery disease and MI are still the leading causes of death in the western world. The currently available pharmaceutical therapy is often not effective and a large number of patients are not suitable candidates for coronary revascularization procedures. Thus, it is imperative that we develop novel treatments for myocardial ischemia.

5.2. Why do we need to identify new pathways involved in angiogenesis?

One approach to treat myocardial ischemia is to enhance blood flow locally to the area of ischemic insult by promoting angiogenesis. Basic research on the fundamental physiological mechanisms of blood vessel development and formation has led to the discovery of multiple angiogenic growth factors and inhibitors (11, 125, 126). To date, several anti-angiogenic therapies are available for clinical use in the treatment of cancer (127, 128), and in clinical trials to treat age-related macular degeneration (129). However, although pro-angiogenic therapies (e.g., VEGF and FGF-2) showed initial promise in animal models and in small uncontrolled pilot studies in patients with ischemic heart disease and peripheral arterial occlusive disease (130, 131), clinical efficacy has not been unequivocally reported (132).

There are a number of potential explanations for the disappointing results obtained in clinical trials, including poor study design, inadequate mode of drug delivery and lack of cell-specific targeting. In addition, studies in animals and humans have shown that delivery of a single angiogenic agent is not sufficient to promote functional and stable angiogenesis, and overexpression of some growth factors can cause serious complications (133-136). In mice, for example, forced VEGF expression in myocardium has been shown to cause heart edema and formation of hemangioma, which may contribute to heart failure and even death (136). These vascular complications are caused mainly by the instability and leakiness of the newly formed vascular networks. As the establishment of stable and functional blood vessel networks is a complex process that requires several angiogenic factors to stimulate vessel sprouting and remodeling of the primitive vascular network (134), it is likely that a combination of angiogenic growth factors is required to enhance the angiogenic efficacy (137, 138).

Many unanswered questions thus still remain, such as which angiogenic factor or combination of factors should be administered and how should they be administered. Further clarification of the cellular and molecular mechanisms underlying angiogenesis could therefore lead to a more rational design of therapeutic angiogenic strategies.

5.3. Why did we investigate hypoxia-induced angiogenesis?

It is well established that hypoxia regulates a variety of genes that affect a myriad of cellular processes, including metabolism, cell survival, oxygen delivery and angiogenesis (139). Chronic hypoxia has been known for many years to protect against death from ischemic heart disease, but the mechanisms involved are poorly understood. During MI, hypoxia activates multiple signaling pathways in an attempt to minimize cellular injury and maintain cardiac output, and the promotion of angiogenesis may play a key role in this protection. The link between hypoxia and the regulation of angiogenesis is an area of intense research, and it is essential that we further elucidate the underlying cellular and molecular mechanisms to identify key targets that could be ultimately exploited for the treatment of ischemic heart disease.

In this thesis, we investigated the effects of hypoxia on cardiac angiogenesis using two mouse models: a model of systemic hypoxia and a model of MI. In our model of systemic hypoxia, pretreatment with hypoxia for three weeks before MI resulted in increased coronary blood flow and capillary and arteriolar density. In addition, in our model of MI, we observed infiltration of capillaries and small arterioles into the core of the infarcted ventricles one week after MI. Thus, both models are suitable to investigate the cellular and molecular pathways involved in hypoxia-induced angiogenesis.

5.4. What are the novel findings described in my thesis?

5.4.1. Chronic hypoxia induces increases in SUMO-1, which may stabilize HIF-1α to promote angiogenesis

We showed that SUMO-1 levels in mouse brain and heart increase after exposure to chronic hypoxia, and that SUMO-1 interacts with HIF-1α in response to hypoxia. Thus, we propose that hypoxia-mediated increases in SUMO-1 expression could participate in the modulation of HIF-1α function.

It is well established that HIF-1α regulates transcriptional activity of many genes involved in a number of cellular processes, including angiogenesis, cell survival and metabolism. A number of studies support a potential protective role of HIF-1α in cardiac ischemia. For example, HIF-1α overexpression in the mouse heart reduces infarct size and improves cardiac function after MI (140). Stabilization of HIF-1α with PR39, a macrophage-derived peptide, has been shown to increase peri-infarct vascularization in MI mice (26). Dimethyloxalylglycine, which also stabilizes HIF-1α, increases VEGF production and capillary density in a mouse model of hindlimb ischemia (141). HIF-1α is sumolyated by SUMO-1 in hypoxia, which results in increased HIF-1α stability and transcriptional activity (124). It is, therefore, possible that the hypoxia-mediated increases in SUMO-1 observed in mouse heart could promote angiogenesis by stabilizing HIF-1α activity.

5.4.2. Chronic hypoxia protects the heart from MI by promoting angiogenesis: role for PDI?

We showed that exposure to chronic hypoxia for three weeks before MI improves myocardial function and coronary blood flow, prolongs survival, reduces infarction size and increases capillary and arteriolar density in the myocardium in mice. We also demonstrated increased expression of PDI in vascular endothelium after chronic hypoxia. Inhibition of PDI in endothelial cells in vitro resulted in increased apoptosis and reduced cell migration and adhesion, indicating that PDI may play an integral role in angiogenesis.

How could PDI promote angiogenesis? PDI has been shown to co-localize and interact with the endothelial adhesion molecule (142) $\alpha_v\beta_3$ integrin at the surface of activated endothelial cells (143). PDI may thus promote cell migration and adhesion, key stages in the angiogenic process, by inducing conformational changes in $\alpha_v\beta_3$ integrin (143).

5.4.3. Hypoxia promotes endothelial cell proliferation by activating the endoglin/ALK-1/SMAD1/5 but not the endoglin/ALK-5/SMAD3 signaling pathway

We showed that hypoxia promotes endothelial cell proliferation by activating the endoglin/ALK-1/SMAD1/5 but not the endoglin/ALK-5/SMAD3 signaling pathway in vitro.

Although it is well known that TGF-β is a pro-angiogenic factor (34), an aspect that has puzzled researchers for years is the bifunctional effect of TGF-β on endothelial cells (73). Whereas TGF-β-mediated activation of the ALK-1/SMAD1/5 pathway stimulates the proliferation and migration of endothelial cells, TGF-β-mediated activation of the ALK-5/SMAD2/3 pathway inhibits these processes (70, 75, 144). Thus, it seems that expression of genes downstream of TGF-β may be modulated in endothelial cells differently under certain circumstances.

We thus propose that hypoxia preferentially activates the ALK-1/SMAD1/5 pathway by increasing endoglin expression in the endothelial cells. Thus, the induction of this signaling pathway represents a potential mechanism for regulation of angiogenic responses in myocardial remodeling after MI.

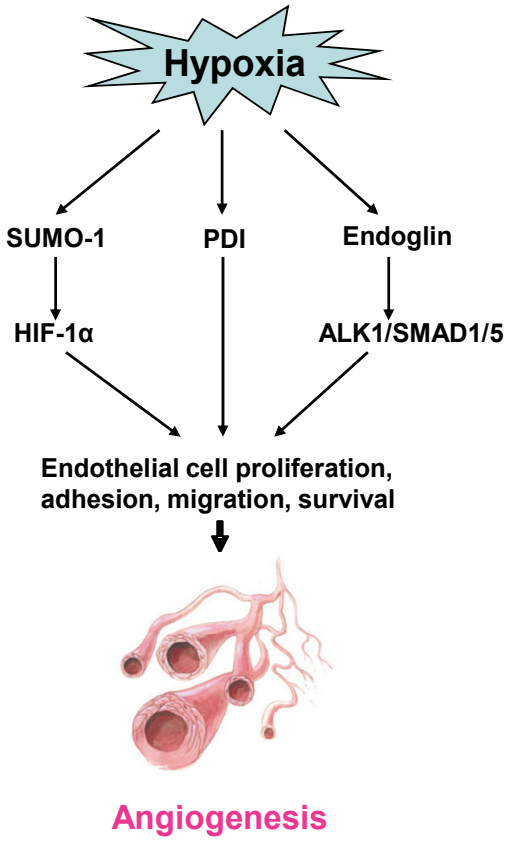

Figure 4. *SUMO-1 stabilizes HIF-1α, which stimulates endothelial cell proliferation by activating pro-angiogenic genes. PDI promotes endothelial cell adhesion, migration and survival. Endoglin promotes endothelial cell proliferation through the ALK-1/SMAD1/5 signaling pathway.*

5.5. Concluding remarks

Promotion of angiogenesis is a promising therapeutic strategy for the treatment of ischemic cardiac disease, but further clarification of the cellular and molecular mechanisms involved is required to determine the optimum therapeutic approach. In this thesis, we have made advances in understanding the complex network of molecules and signaling pathways that link the action of hypoxia to the protective response of angiogenesis (Figure 4). Further research is, of course, required to translate the significance of these findings from the mouse into humans. However, basic research in this field is an essential step to identify key proteins and signaling pathways that can be targeted in the future to counteract the devastating consequences of ischemic heart disease.

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