Determination of the Cardiovascular Phenotype of Different Transgenic Mouse Models

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For my family and my girlfriend Silja.

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3T3	=	murine fibroblast cell line
ACE	=	angiotensin converting enzyme
AUG–codon	=	adenine-uracil-guanine codon for translation initiation
BSA	=	bovine serum albumin
CCR2	=	chemokine receptor 2
cDNA	=	complementary deoxyribonucleic acid
CHF	=	congestive heart failure
CK	=	creatine kinase
CMV	=	cytomegalovirus
CT	=	C-terminal tail
CUG–codon	=	cytosine-uracil-guanine codon for translation initiation
DNA	=	deoxyribonucleic acid
EC	=	extracellular domain
*Eco*RI	=	restriction endonuclease
EGF	=	endothelial cell growth factor
ESAF	=	endothelial cell-stimulating factor
FCS	=	fetal calf serum
FGF–1	=	fibroblast growth factor 1
FGF–2	=	fibroblast growth factor 2
FGFR	=	fibroblast growth factor receptor
FGFR1	=	fibroblast growth factor receptor 1
FGFR2	=	fibroblast growth factor receptor 2
FGFR3	=	fibroblast growth factor receptor 3
FGFR4	=	fibroblast growth factor receptor 4
FGFs	=	fibroblast growth factors
g	=	gravitation constant
HIF–1	=	hypoxia inducible factor 1
HIF–1α	=	hypoxia inducible factor 1α
HIF–1β	=	hypoxia inducible factor 1β

i.p.	=	intra peritoneal
ICAM–1	=	intercellular adhesion molecule 1
IG	=	immunoglobulin
IGF–1	=	insulin-like growth factor 1
IL–1	=	interleukin 1
IL–2	=	interleukin 2
IL–6	=	interleukin 6
IL–8	=	interleukin 8
IU	=	international units
JE	=	murine homologue of MCP–1
JM	=	juxtamembrane domain
kb	=	kilo base
KD	=	kinase domain
kDa	=	kilo Dalton
LDH	=	lactate dehydrogenase
LDI	=	laser doppler imaging
LDL	=	low density lipoprotein
MAP kinase	=	mitogen-activated-protein kinase
MCP–1	=	monocyte chemoattractant protein 1
MLC2v	=	ventricular isoform of the regulatory myosin light chain
MMPs	=	matrix metalloproteinases
MRI	=	magnetic resonance imaging
mRNA	=	messenger ribonucleic acid
NIH	=	National Institutes of Health
NO	=	nitric oxide
NOS	=	nitric oxide synthase
NTG	=	nontransgenic control mice
NYHA	=	New York Heart Association
PAOD	=	peripheral arterial obstructive disease
PC	=	phosphatidylcholine
PDGF	=	platelet derived growth factor
PKC	=	protein kinase C

P_{O_2}	=	oxygen partial pressure
rpm	=	rotations per minute
SEM	=	standard error of the mean
SMCs	=	vascular smooth muscle cells
SV40	=	Simian virus 40
T_1	=	longitudinal relaxation time
T_2	=	transversal relaxation time
TG	=	transgenic mice
TGF–β	=	transforming growth factor β
TGF–β_1	=	transforming growth factor β_1
Tie-2	=	tyrosine kinase with immunoglobulin and EGF–homologous regions
TIMP–1	=	tissue inhibitor of metalloproteinase 1
TM	=	transmembrane domain
TNF–α	=	tumor necrosis factor α
VCAM–1	=	vascular adhesion molecule 1
VEGF	=	vascular endothelial growth factor

1. Abstract

Background: Fibroblast growth factors 1 and 2 (FGF–1 and FGF–2), potent mitogens for endothelial cells and vascular smooth cells, are implicated in arterial and capillary growth as well as in cardioprotection. Monocyte chemoattractant protein 1 (MCP–1) is involved in various inflammatory conditions. Utilizing transgenic mice (TG) overexpressing FGF–1, FGF–2, or MCP–1 and nontransgenic controls (NTG), the effects of these factors on vascular development, cellular protection, cardiac performance, and exercise tolerance were studied.

Methods: **1.** Ventricular cardiac myocytes of hearts of FGF–1 TG and NTG were isolated and submitted to simulated ischemia and reoxygenation. The releases of CK and LDH were quantified. **2.** The coronary flow of the hearts of FGF–1 TG and NTG was quantified utilizing *ex vivo* retrograde perfusion under maximal vasodilation at four different pressures. **3.** Right femoral arteries of FGF–2 TG and NTG were occluded. Mice were assigned to a sedentary or a trained group. After 5 weeks, collateral dependent blood flows to the foot and to the gastrocnemius muscle were determined. Exercise capacity was accessed, postmortem angiograms and histomorphometry of collateral arteries were performed. **4.** Exercise tolerance of MCP–1 TG and NTG was determined by graded exercise tests over a period of four months. **5.** The relationship between recovery of exercise capacity and increase in collateral dependent blood flow after bilateral femoral artery occlusion was investigated in three different mouse strains.

Results: **1.** CK and LDH release of myocytes of FGF–1 TG was reduced at 4h and 8h of simulated ischemia. **2.** The pressure dependent increase in coronary flow was markedly elevated in hearts of FGF–1 TG. **3.** Foot and gastrocnemius blood flows as well as exercise capacity were increased in trained FGF–2 TG that showed the formation of a dense collateral network. **4.** Exercise tolerance of MCP–1 TG was markedly reduced. **5.** Increased collateral dependent foot blood flow is only partially reflected by the recovery of exercise capacity.

Conclusions: Cardiac-specific FGF–1 overexpression protects ventricular myocytes against simulated ischemia and increases coronary flow. General FGF–2 overexpression and training enhance the formation of a dense collateral network, increase collateral dependent blood flow and exercise capacity. Cardiac-specific MCP–1 overexpression induces myocarditis and causes exercise intolerance as a typical symptom of congestive heart failure. Recovery of collateral dependent resting blood flow partially increases exercise capacity.

2. Introduction

At the Max-Planck-Institute, Department of Experimental Cardiology, we are investigating possible ways to protect tissues like myocardium and skeletal muscle, which are exposed to ischemia caused by occlusion of a major artery. Protection of ischemic tissues can be achieved by two different mechanisms.

The first mechanism concentrates on the short-term protection of ischemic tissues and includes the phenomenon of ischemic preconditioning. It is well known that a series of brief periods of ischemia induces an intracellular signaling cascade involving protein kinase C and mitogen activated protein kinases. This signaling cascade causes transcriptional events, which finally increase the tolerance of the tissue towards a longer period of ischemia. The application of different growth factors including FGF–1 and FGF–2 before a longer period of ischemia mimics the phenomenon of ischemic preconditioning.

The second mechanism involves the growth of blood vessels that are capable of conducting blood flow to the ischemic tissues. Arteriogenesis, the growth of preexisting collateral arterioles by mitotic cell division and after DNA replication of endothelial and smooth muscle cells, is thought to be the only functionally relevant process to increase blood flow to the ischemic tissues. Arteriogenesis is induced by occlusion of a major artery and serves as a rescue mechanism for the distal tissues.

Tissue ischemia is tolerated only for a short period of time whereas arteriogenesis requires at least one day to become functionally relevant. Therefore, we investigated whether the stimulation of short-term tissue protection could bridge the gap between the time requirements from the onset of ischemia to the increase in collateral dependent blood flow.

Furthermore, we studied possible ways to increase the collateral blood flow to the ischemic tissues. Therefore, it was necessary to explore the mechanisms of vessel growth in general and of preexistent collateral arterioles in particular.

The ideal approach would be to increase the short-term protection and at the same time enforce and accelerate the long-term protection via stimulation of vessel growth to the ischemic tissues possibly by the same growth factor or by combined treatments. Increasing the tolerance towards ischemia and restoring blood flow as fast as possible could reduce or even prevent tissue necrosis and potentially safe hearts, brains, limbs, and therefore, many lives in the Western society.

The studies presented here relate to these two important topics and mainly utilize transgenic mouse models. These studies contribute to other investigations considering the action of various growth factors and chemokines by providing information about some important and unknown features.

Using our own system of cardiac-specific overexpression of FGF–1, we investigated the cardioprotective effect of FGF–1 on isolated ventricular cardiac myocytes subjected to different periods of simulated ischemia and subsequent reoxygenation.

We studied the effects of cardiac-specific overexpression of fibroblast growth factor 1 (FGF–1) on the development of coronary arteries and arterioles. Therefore, we determined the coronary flow using *ex vivo* perfusion of the coronary system under maximal vasodilation at four different pressures.

To explore the effects of general overexpression of FGF–2 on the development of collateral arteries, we occluded the femoral arteries of FGF–2 transgenic and nontransgenic animals. In addition, a sub-population of each group was submitted to regular endurance training to determine if there is any treatment interaction between FGF–2 application and endurance training.

We accessed the effects of cardiac-specific overexpression of monocyte chemoattractant protein 1 (MCP–1) on the development of congestive heart failure (CHF) due to myocarditis. Exercise intolerance as a common symptom of congestive heart failure was determined by graded exercise tests over the period of four months. We explored whether the exercise capacity could serve as an indicator for the decreased cardiac function in this animal model.

Furthermore, we investigated if exercise capacity serves as a physiological marker of the functional capacity of hindlimbs with femoral artery occlusion. For this purpose we utilized three strains of mice with different dynamics in the recovery of collateral dependent blood flow and determined the recovery of exercise capacity after femoral artery occlusion. Because arteriogenesis displays different dynamics in these mouse strains we anticipated changes in the functional capacity of the hindlimbs and thus, in exercise capacity.

3. Review of Literature

3.1. FGFs in the Cardiovascular System

3.1.1. Biochemistry of FGF–1 and FGF–2

As early as 1939, tissue extracts of brain were shown to be a rich source of factors that promoted fibroblast proliferation *in vitro* (for review see [1]). In the following years, acid extracts of bovine pituitary were found to contain a potent polypeptide mitogen for the murine cell line 3T3 and for chondrocytes. The protein was distinct biochemically from other known hormones or growth factors and extraction conditions seemed to be important for the maintenance of the biological activity.

In the 1970s, a basic polypeptide mitogen for 3T3 cells and mesoderm- and neuroectoderm-derived cells was isolated from bovine pituitary and brain. This polypeptide was named fibroblast growth factor. However, due to technical difficulties its structure could not be analyzed. Later, these difficulties were overcome and it turned out that acidic and basic fibroblast growth factors are related polypeptides and serve to establish the basis for a larger family of polypeptide growth factors. Both FGFs were also shown to be potent mitogens for endothelial cells. Because more growth factors belonging to the FGF–family were discovered the nomenclature FGF–1, FGF–2, etc. has been chosen. Acidic and basic fibroblast growth factors were assigned the names FGF–1 and FGF–2, respectively.

Before the structures of FGF–1 and FGF–2 were identified and the present nomenclature was chosen other names have been ascribed to these FGFs. Among them are eye-derived growth factor 1 and –2, endothelial cell growth factor, heparin–binding growth factor α and –β, anionic and basic brain–derived growth factor, hypothalamus-derived growth factor, retinal–derived growth factor, astoglial growth factor 1 and –2, cartilage–derived growth factor, myogenic growth factor, pituitary growth factor, bone–derived growth factor, anionic endothelial growth factor, prostatic growth factor, and prostatropin. The variety of names describing their source or target cells demonstrate the potential biological significance of FGF–1 and FGF–2.

In 1986, the primary structure of human FGF–1 derived from the cDNA clone was identified [2]. This study demonstrated that there is a single copy of the

FGF–1 gene and that it is located at chromosome 5 at bands 5q31.3 to 5q33.2. The gene encodes for a 4.8 kb mRNA. It was shown that the open reading frame of FGF–1 is flanked by translation stop codons and does not contain a signal peptide or a hydrophobic domain for the secretion of FGF–1. Later, the same was shown to be true for FGF–2. Sequence analysis of bovine and human FGF–1 revealed an approximate 92% sequence identity, conservation of the positions of two of three cysteine residues, and the presence of a potential N–linked glycosylation site. Structural analysis of FGF–1 and FGF–2 demonstrated a 55% homology between the two polypeptides [3]. Even though the FGF–2 gene is located at chromosome 4, the structural similarity together with similar organ sources and biological activities suggest FGF–1 and FGF–2 may have originated from a common ancestral gene.

Both genes are similar in their overall organization. They contain three exons separated by two relatively large introns. The major difference that appears after analysis of the cDNAs is the precise location of the amino terminus of the two proteins. The termination codons flanking the open reading frame of the FGF–1 gene were not found in the FGF–2 gene.

FGF–2 was first identified as a 146 amino acid protein isolated from bovine pituitary [4]. The isolated form possessed a strong potency to stimulate the proliferation of bovine aortic endothelial cells. This effect was markedly inhibited by acidic purification procedures. Therefore, it was commonly referred to as “basic” FGF.

Cloning of FGF–2 cDNA lead to a predicted length of 155 amino acids. However, only the 146 amino acid protein and higher molecular weight forms (196, 201, 210 amino acids) were actually isolated from tissues. The shorter form is derived from the 155 amino acid protein by proteolytic degradation [5]. Longer forms are generated by initiation of translation at different CUG–codons located 5’ to the AUG–codon, which is used for translation of the 155 amino acid protein [6].

The three forms initiated at CUG-codons have molecular masses of 22, 22.5, and 24 kDa and are predominantly located in the nucleus. The AUG-initiated form with a molecular mass of 18 kDa is primarily localized in the cytoplasm. However, the occurrence of the different forms may depend upon the specific cells examined and the expression levels of FGF–2.

The 18 kDa form of FGF–2 contains 12 anti–parallel β–sheets organized into a trigonal pyramidal structure. Amino acid residues 13–30 and 106–129 are believed to be involved in receptor binding [7]. Serine 64 and threonine 112 are

potential phosphorylation sites, which can be phosphorylated by protein kinase A and protein kinase C, respectively [8]. Even though FGF–2 contains four cysteine residues, there are no intramolecular disulfide bonds.

The high molecular weight forms of FGF–2 have an amino terminal extension as a unique feature. The 24 kDa form contains nine glycin–arginine residues, of which seven are methylated [9,10]. However, neither the exact number nor the functional significance of the methylated arginines of the high molecular weight forms is known, but they might be involved in nuclear transport or retention.

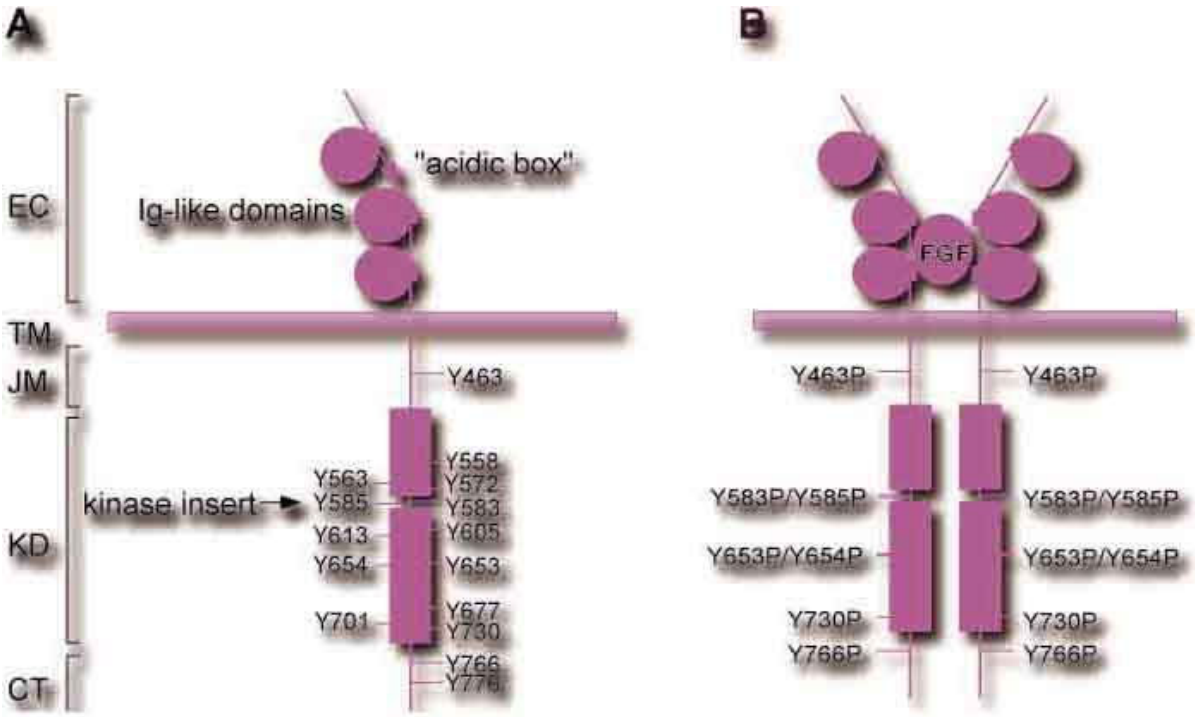


Figure 1. *Schematic structure of FGFR1.* ***A.*** *The overall structural organization is similar for the four FGF receptors. The extracellular domain (EC) contains two or three immunoglobulin(Ig)-like domains, followed by the transmembrane (TM) stretch, the juxtamembrane (JM) domain, the kinase domain (KD) interrupted by a short kinase insert, and a C–terminal tail (CT). The positions of the tyrosine residues in the intracellular domain are indicated. The acidic box indicated in the intracellular domain is a specific feature of FGF–receptors.* ***B.*** *The positions of identified phosphorylatable tyrosine residues are indicated in the ligand-bound, dimerized FGFR1. Tyrosine phosphorylation sites in other FGF receptors remain to be determined. However, FGFR2, FGFR3, and FGFR4 all contain tyrosine residues corresponding to tyrosines 653, 654, 730, and 766 in FGFR1 (reprinted from [11]).*

FGF–1 and FGF–2 interact with specific cell surface receptors, of which four major families have been identified; FGFR1, FGFR2, FGFR3, and FGFR4. These receptors share common features like a conserved cytoplasmic tyrosine kinase

domain, a transmembrane domain, and an extracellular ligand-binding domain (Figure 1). The composition of the ligand-binding domain differs between the splice–variants. They contain either two or three immunoglobulin(Ig)–like loops. The existences of additional splice variants with sequence modifications in the intracellular portions of the protein have been demonstrated (for review see [11]).

FGF binding induces autophosphorylation of the receptor and receptor association with putative substrates such as phospholipase C–γ. The long–term mechanism of FGF–1 and FGF–2 action involves growth factor translocation to the nucleus. After at least 12 hours of stimulation with FGF–1, the maximal DNA synthesis of 3T3 cells was achieved [12]. In stimulated cells, the FGFR1 was phosphorylated and translocated perinuclearly. At the same time FGF–1 accumulated in the nucleus. Therefore, it was concluded that signaling through the FGF receptor might occur at the plasma membrane and after receptor translocation to an intracellular site.

However, receptor-independent mechanisms for FGF–1 stimulation of proliferation have also been proposed [13]. FGFR expression levels change markedly and during proliferation, differentiation, or malignant transformation. FGFR expression levels during differentiation for example differ depending on the experimental conditions and cell types used and therefore, no general conclusion about FGFR expression levels can be derived.

3.1.2. Cardiovascular Background

Ischemic heart disease caused by stenosis or occlusions of the coronary arteries represents the major cause of mortality in western societies. Two distinct physiological processes can help in maintaining the viability of ischemic myocardium: the development of an effective vascular collateral system, and the effect of myocardial preconditioning, the endogenous protection of cardiac myocytes against ischemia.

During the last decade, extensive research has been dedicated to the basic mechanisms responsible for the formation and enlargement of blood vessels in ischemic tissues. It was mainly focused on the role of growth factors as modulators of vascular growth due to their capacity for directing the proliferation and differentiation of cells composing the vascular system. FGF–1, FGF–2, vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF–1),

platelet derived growth factor (PDGF), and angiopoietins have received special attention.

FGF–1 and FGF–2 are potent angiogenic factors inducing capillary formation. Their mitogenic capacity for endothelial cells is even stronger than that of the endothelial specific mitogen VEGF (for review see [14]). Additionally, both FGF–2 and VEGF have been demonstrated to act synergistically in vitro [15] and in vivo [16]. Unlike VEGF, FGF–1 and FGF–2 also induce vascular smooth muscle cell (SMC) proliferation and migration in cell cultures [17], constituting exceptional candidates for the regulation of vascular development and growth after an episode of ischemia.

FGFs are members of the heparin binding family of growth factors. Due to their high affinity to heparin, FGF–1 and FGF–2 commonly accumulate in the extracellular matrix. They bind to heparin sulfate proteoglycans, which aid in the protection against protein degradation. Indeed, with a half lifetime of about 15 minutes, unbound FGF–1 is very rapidly degraded by proteases [18].

FGF–1 and FGF–2 proteins lack a signal peptide sequence for secretion from cells [19]. Their secretion *in vivo* is still not entirely clarified. In the normal heart, FGF mRNA has been shown to be expressed by cardiac myocytes, and the protein located to both myocytes and extracellular matrix [20,21]. In contrast, FGF receptors are normally not expressed by cardiac myocytes and endothelial cells [22]. However, FGF receptor and ligand have been demonstrated to be upregulated during ischemia in several animal models [23,24]. These observations have led to the hypothesis that a tight regulation of FGF receptors might be a basic mechanism for the control of vascular growth during ischemia. This type of regulatory mechanism has been shown to be important for tumor angiogenesis [25]. FGF protein bound to heparin sulfate proteoglycans and stored in the extracellular matrix might serve as a growth factor reservoir, ready to bind its receptor on endothelial cells and SMCs under ischemia-induced upregulation.

3.1.3. FGFs in Therapeutic Angiogenesis

There is accumulating evidence from studies, in which the induction of angiogenesis for therapeutic purposes is investigated, demonstrating the importance of FGF–1 and FGF–2 for the vascular response against ischemia. In these in vivo experiments, native, recombinant, or vectors coding for the FGF protein are locally or systemically infused in animals subjected experimentally to

ischemia. Two main experimental designs have been used. The model of chronic myocardial ischemia, in which one branch of the coronary arterial tree is gradually stenosed by ameroid constrictors, was mainly developed in dogs and pigs. The model of acute hindlimb ischemia, in which the femoral artery is chirurgically excised or occluded, was designed for rabbits.

The first study on the therapeutic potential of FGF in the ischemic heart was conducted by Banai and collaborators utilizing the dog model [26]. Locally delivered FGF–1 via a soaked sponge induced SMC hyperplasia in arterioles and small arteries in the area of the subendocardial infarction. No effect on capillary growth was detected.

In a more recent study, López et al. used the porcine model of chronic left circumflex artery occlusion by Ameroid constrictors to test the effect of FGF application [27]. In this study FGF–1 together with heparin were administered perivascularly via sustained release from a polymer matrix. The matrix was implanted in the course of the occluded left circumflex coronary artery. Treated animals showed significantly improved myocardial blood flow in the compromised territory under resting coditions and during pacing as well as enhanced global and regional left ventricular function. However, the study did not provide conclusive evidence on how FGF–1 released at the epicardium can induce collateral vessel growth in the endocardium where it usually occures in the pig.

Other experiments with the model of chronic myocardial ischemia have also demonstrated improved myocardial function and collateral flow, as well as new vessel formation after local or systemic administration of FGF–2 [28-31].

Similar results have been obtained with the rabbit model of hindlimb ischemia. Administration of FGF–1 intramuscularly [32], or of cDNA coding for a secreted form of FGF–1 systemically [33] resulted in improved perfusion, increased number of capillaries, and augmented angiographically visible collateral arteries (for other studies of therapeutic FGF application see chapter 3.3.8).

These studies provide evidence that FGF–1 and FGF–2 play important roles in the response of hypoxic tissues to ischemia, increasing blood flow, and improving muscular function. However, the mechanisms implicated in these effects of FGF–1 and FGF–2 are still unknown. Several distinct physiological processes may increase blood flow, namely the angiogenic response of the capillary system, de-novo formation of arterioles, and the growth of collateral arteries. Improvement in ventricular function might be the consequence of increased blood

flow, but it could also result from a hypothetical cardioprotective potential of FGF–1 and FGF–2.

3.1.4. FGFs in Vascular Response Against Ischemia

Angiogenesis is defined the growth of capillary tubes from pre-existing capillary vessels or small venuoles [34]. Single endothelial cells proliferate, disrupt the continuity of the vessel wall, invade the surrounding tissue, and form new tubular structures, which expand the pre-existing capillary bed.

FGF–1 and FGF–2 induce capillary tube formation *in vitro* and are able to increase the capillary density of ischemic organs *in vivo*. However, it is currently believed that capillaries are not able to conduct enough blood flow to ischemic tissues of larger sizes, when the stenosis affects the perfusion of a large tissue mass.

Resistance vessels are the major determinants of blood flow, and therefore they are good candidates for increasing the blood flow to an ischemic tissue. Very little is known about the growth of arteries in adult organs. De novo formation of arteries takes place by elongation of the distal segment of terminal arterioles [35]. The capillary to which an arteriole is connected must recruit new SMCs from surrounding pericytes or from SMCs in the distal end of the terminal arteriole, and differentiate into a histologically mature artery [35]. This process requires pericyte and SMC proliferation, differentiation, and matrix deposition, resembling the embryonic or neonatal process of arterial differentiation.

The increased number of angiographically detectable vessels in the rabbit model of hindlimb ischemia obtained after treatment with FGF–1 may represent this type of growth [33]. In this experimental model, the whole femoral artery is excised, so that the only source of blood to the ischemic muscles are capillaries as well as small arteries that can grow into the ischemic tissue from nearby arteries not affected by exision of the femoral artery.

Collateral arteries are vascular segments, which connect two feeding arteries. The anatomical and hemodynamical characteristics of collateral arteries make them a unique vascular resource against ischemia. Upon stenosis of a major artery, the collateral arteries are the only vessels able to bridge the occluded segment, providing an effective blood supply to the ischemic tissue. Likewise, collateral arteries are the only vascular structures that experience a change in

shear stress, caused by the pressure difference after an arterial occlusion. This increase in shear stress has a known potential effect as a vascular growth promoter. Moreover, after occlusion of an arterial segment, collateral arteries begin a process of growth, called arteriogenesis that culminates in a 20 fold increase in their final diameter, improving their flow capacity by the factor of 10, and leading to a final compensation up to 30% of the normal maximum flow [24,36-38]. Currently, it is thought that only the growth of collateral arteries by arteriogenesis is able to provide enough of blood flow to the ischemic area.

With this knowledge we planned a study with mice generally overexpressing FGF–2. We recently established a mouse model of collateral artery development after occlusion of the femoral artery. We hypothesized that general overexpression of FGF–2 would facilitate arteriogenesis.

3.1.5. FGFs in Arteriogenesis

The process of arteriogenesis consists of complete reorganization of the collateral vessel wall, including the destruction of the old lamina media, and formation of a new one. The first morphological evidence of collateral growth is the activation of endothelial cells, probably induced by shear stress. They express cell adhesion molecules for the attachment and migration of monocytes [39]. Once they had migrated into the arterial wall, the activated macrophages express cytokines and growth factors, including FGF–2 [40]. These substances are most probably the responsible signals that trigger the whole process of arteriogenesis. Moreover, gene expression experiments performed in our laboratory with the rabbit model of femoral artery occlusion have shown a significant up-regulation of the FGFR1 mRNA during the first phase of collateral growth [41]. The FGFR1 is the predominant form of the four known FGFRs expressed by proliferating SMCs [42], suggesting that FGFR1 mediates signal transduction in proliferating SMCs, and pointing to a crucial role of FGFs in the initial phase of arteriogenesis.

During this initial phase of growth, the internal elastic lamina of collateral arteries is degraded, and SMCs dedifferentiate, proliferate and migrate to the subendothelial space, forming a thick neointima, which is required to build a new vessel [43,44]. These SMCs show an undifferentiated or synthetic phenotype with abundant organelles, loss of basement membrane, reduced amount of α–smooth muscle actin, vinculin, caldesmon, and calponin. The intermediate filaments of these cells change from desmin to vimentin during the period of migration and

growth. The extracellular matrix of growing collateral arteries shows a deep rearrangement, with increased collagen IV and fibronectin secretion. All these characteristics indicate an extensive vascular remodeling process, probably regulated by FGFs that resembles the embryonic development and differentiation of arteries.

The embryonic development of arteries is a complex developmental process, in which multiple molecular signals are implicated. Numerous homebox genes and transcription factors have been identified during the last years, but their particular functions still remain unknown. The system Tie–2/angiopoietin–1 has been shown to participate in the regulation of the process [45], whereas PDGF probably controls the secretion of extracellular matrix and the cellular differentiation (for review see [46]). FGF is also implicated in the embryonic development of arteries [47], and it is probably necessary for the cellular proliferation and differentiation of SMCs.

Recently, a strain of transgenic mice that specifically overexpress FGF–1 in the heart was developed in our laboratory [48]. The gross anatomy, histological structure and ultrastructure of the hearts of these transgenic mice are normal. However, a significant 1.5 fold increase in the density of small arteries was noted, whereas the capillary density was unchanged. Moreover, the number of branches of the main coronary arteries was also significantly increased 1.5 fold. The arrangement and anatomical disposition of the coronary arterial tree was normal. These results suggest that FGF–1 is a master regulator of the development and growth of the arterial system. The cardiac–specific overexpression offers an appropriate animal model for the study of potential genes involved in the development and growth of arteries in the heart during embryogenesis and during ischemic heart disease.

The increase in the density of small arteries and in branching of the main coronary arteries could have potential effects on myocardial flow. Therefore, we determined the coronary flow using *ex vivo* retrograde perfusion of the heart under maximal vasodilation at four different pressures.

3.1.6. FGFs in Myocardial Ischemic Preconditioning

Ischemic preconditioning is defined as the cardioprotective effect against ischemia obtained after repetitive short-term coronary occlusions [49]. After one or more short cycles of ischemia, infarct development is delayed, but not prevented.

Thus, preconditioning can limit infarct size during a temporary occlusion, but not during a permanent occlusion.

During the last years, enormous efforts have been made to identify the factors responsible for cardioprotection. The aim of many of these studies was to discover trigger factors, which could be released at the onset of ischemic preconditioning, looking for new and efficient therapies against myocardial infarction. Adenosine, acetylcholine, catecholamines, angiotensin, bradykinin, endothelin, and opioids are some of these triggers (for review see [50]). The receptors of some of these factors are coupled to G–proteins. It has been proposed that their activation may converge in a common pathway, resulting in protein kinase C (PKC) activation, which would be responsible for cardioprotection [51]. Indeed, ischemic preconditioning can be selectively blocked by PKC inhibitors [52,53].

Other studies have suggested that kinases other than PKC are implicated in the mechanism of ischemic preconditioning [54,55]. Previous studies in our laboratory demonstrated up–regulation of tyrosine receptor kinase–ligands following brief cycles of coronary occlusion [56]. FGF–1 was one of these ligands. In neural tissue and skeletal muscle models, FGF–1 demonstrated trophic and protective effects [57,58]. With this knowledge, we conducted a study on the effects of FGF–1 and FGF–2 administration before an episode of ischemia.

FGF–1 or FGF–2 was directly infused into the myocardium of pigs just before coronary occlusion [59]. Both FGF–1 and FGF–2 infusion showed a protective effect, which significantly reduced infarct size. In terms of ischemic tolerance, FGF infusion approximately doubled the time required for complete infarction.

The cardioprotection obtained after FGF infusion is probably not a general effect of activation of tyrosine kinases, because other tyrosine kinase receptor ligands, like VEGF, do not achieve protection [59]. On the other hand, suramin, a growth factor antagonist, prevented FGF-induced cardioprotection, indicating that the preconditioning effect of FGF is receptor mediated. Moreover, when the tyrosine kinase inhibitor genistein was infused prior to FGF, cardioprotection vanished, indicating that receptor activation is required for the preconditioning effect of FGF.

One possible explanation of this preconditioning effect relies on the already known vasodilatory capacity of FGF–1 [60], that might lead to improved perfusion. However, infusion of a truncated form of FGF–1, non-mitogenic but

hemodynamically active [60], did not result in cardioprotection, indicating that the preconditioning effect is due to the mitogenic activity of FGFs.

In this set of experiments, FGF–1 showed a significantly stronger preconditioning effect than FGF–2, in terms of protein concentration required to obtain a functional response. This result is surprising because both FGFs have been demonstrated to bind equally to FGFR-1 and FGFR-2 in vitro [61]. However, some studies have stressed the fact that FGF–1 has a greater importance than FGF–2, in terms of gene expression in normal hearts [62,63].

As already mentioned, we have recently developed a transgenic strain of mice specifically overexpressing FGF–1 in cardiac myocytes [48]. Believing that this may be an appropriate model to study the cardioprotective effect of FGF–1, we have performed experiments in which the left coronary artery was chirurgically occluded. The infarct and risk areas were quantified in tissue slices 15, 30, 45, 60, and 75 minutes after coronary artery occlusion [64].

The hearts of transgenic mice showed a significantly delayed infarct development. After 75-90 minutes of occlusion, however, the infarcts had reached the same size in both transgenic and control mice. Most likely, this excludes the possibility that the increased arterial density of transgenic mice, already discussed in this chapter, could cause an improved blood flow that would have markedly reduced the final infarct size.

These results demonstrate that overexpression of FGF–1 is cardioprotective in mice, strengthening the hypothesis of an important role for FGF–1 in ischemic myocardial preconditioning. However, to undoubtedly prove that transgenic cardiac myocytes are protected against ischemia without any influence of the vasculature, we had to conduct a new set of experiments. The best solution for this purpose seemed to be the isolation of ventricular cardiac myocytes and submitting them to simulated ischemia. The results of this study would then prove if overexpression of FGF–1 in cardiac myocytes protects them against ischemia without influence of the vasculature.

The next question arising would concern the pathway by which FGF–1 exerts its cardioprotective function?

FGF ligand binding induces receptor dimerization, autophosphorylation, and initiation of a downstream signaling cascade leading to the activation of mitogen-activated protein kinases (MAP kinases). Interestingly, MAP kinases have been shown to be upregulated by transient ischemia in rats [65]. MAP kinases are mediators of signal transduction to the nucleus, and are involved in the response

to cellular stress such as hypoxia, activating the transcription of genes probably implicated in the cellular responses of the myocardium against ischemia.

Further studies with our FGF–1 transgenic mouse model are needed, in order to explore the functions and pathways of FGF–1 leading to myocardial ischemic preconditioning, as well as in coronary vascular growth during embryonic development, neonatal life, and episodes of ischemia.

3.2. MCP–1 in the Cardiovascular System

3.2.1. Biochemistry of MCP–1

The study of the influence of cardiac-specific overexpression of MCP–1 on exercise tolerance over a period of four month is a relatively small part of this thesis. Therefore, only a short outline about the role of MCP–1 in the cardiovascular system will be provided.

There is accumulating evidence that MCP–1 plays important roles in pathophysiological conditions like decompensated heart failure, myocarditis, atherosclerosis, and inflammation. MCP–1 is a potent chemokine attracting monocytes to the location of its secretion. Most chemokines have a conserved set of four cysteine residues. Chemokines with the amino terminal pair of cysteine residues adjacent to each other are called C–C chemokines. In the case of an amino acid residue between the two cysteines they are called C–X–C chemokines. MCP–1 is considered the most studied member of the former class, whereas interleukin 8 (IL–8) is the best studied member of the latter.

Shortly after the discovery of the neutrophil-specific chemoattractant now called IL–8, different independent groups purified MCP–1 on the basis of its ability to attract monocytes *in vitro*. Surprisingly, the amino acid sequence of MCP–1 indicated that the gene encoding its murine homologue had already been cloned. It was discovered in mouse fibroblasts as a platelet-derived growth factor-inducible gene, called *JE* [66]. This became a recurring theme during the following years as more chemokines were isolated and sequenced. Many cDNAs encoding chemokines had already been isolated by differentially screening activated cells. This was probably due to the fact that the genes of many chemokines are extremely inducible, with expression levels often increasing 30- to 50–fold after stimulation.

The genes encoding for C–C chemokines cluster at chromosome 17 (q11.2–12). The amino-terminal region of MCP–1 is characterized by a short β–sheet (Figure 2), designated β_0. It is otherwise disorganized and floppy, nevertheless essential for the biological activity of MCP–1 and possibly for dimerization [67]. The following β_1–sheet including serine 27, tyrosine 28, and arginine 30 is also important for the activity of MCP–1. Thereafter, a C–terminal α–helix follows, which seems to interact with glycosaminoglycans. This interaction might enhance dimerization and high-affinity receptor binding.

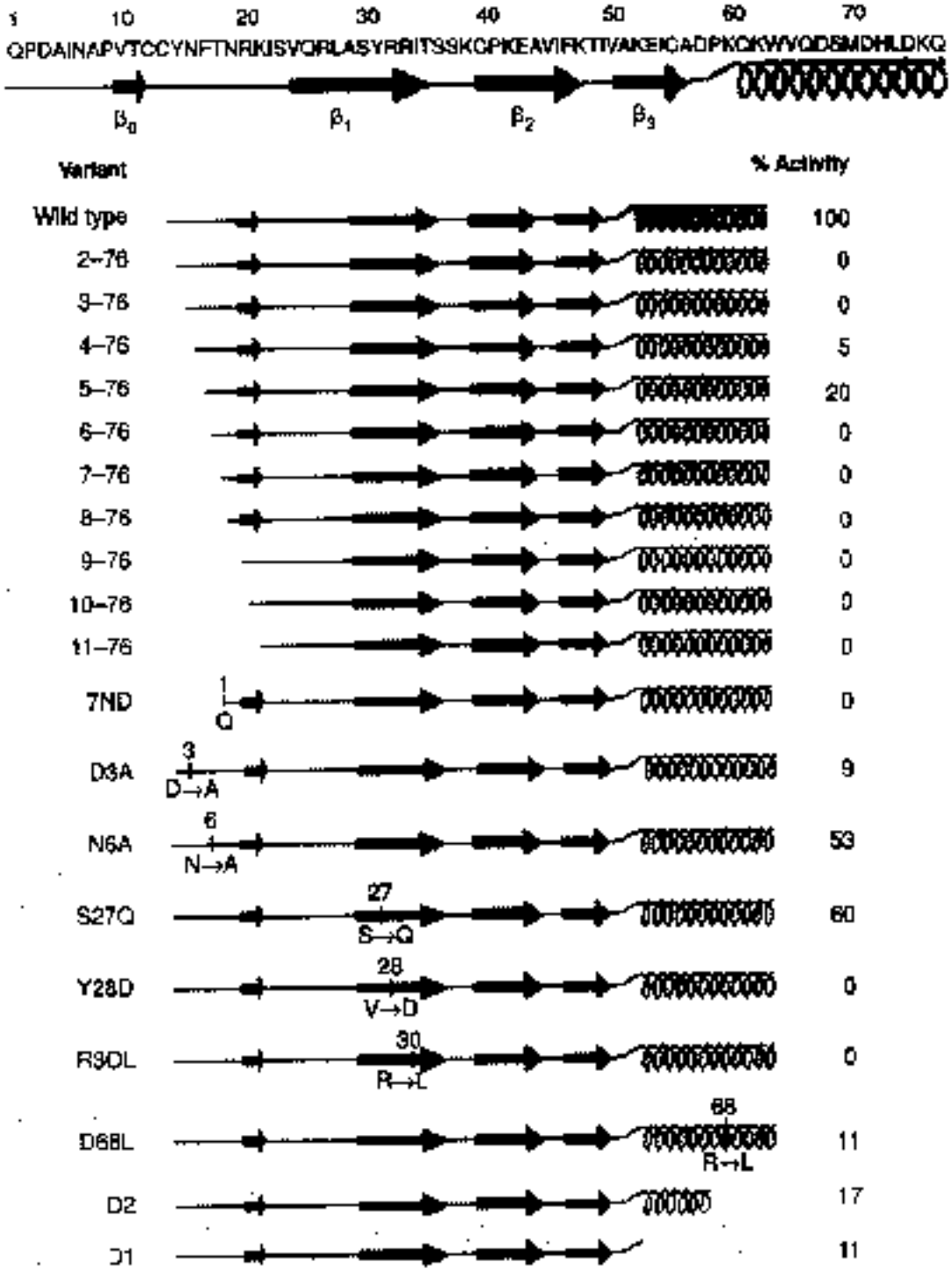


Figure 2. *Structure-function relationships for MCP–1. Indicated immediately beneath the amino acid sequence of human MCP–1 are secondary structural features of the protein, including the four β-sheets (β_0–β_3) and the C-terminal α–helix. Shown below are several experimentally generated MCP–1 variants, including deletion mutants and mutants in which individual amino acids were substituted. The first line for example (2-76) indicates a variant in which the N–terminal amino acid (number 1) was removed, leaving the amino acids 2 through 76. D3A describes a substitution of amino acid 3. Aspartate (D in the single-letter amino acid code) was changed to alanine. The 7ND variant lacks the*

amino acids 2–8. Next to the name of each variant is a schematic of its structure and its relative biological activity (reprinted from [68])

However, the physiological role of MCP–1 dimers remains to be clarified. It has been shown that MCP–1 is under physiological concentrations a monomer and dimerization might happen at concentrations that are irrelevant [69]. However, this does not rule out the possibility that two independent monomers might bind simultaneously to the receptor to generate a signal. It has also been shown that MCP–1 as a dimer interacts with other chemokines even at physiological concentrations [70].

MCP–1 mainly interacts with the chemokine receptor 2 (CCR2), a specific seven-transmembrane-domain G protein coupled receptor on the surface of its target cells.

3.2.2. MCP–1 and Atherosclerosis

The attraction of leukocytes to a specific area of the vasculature and the migration through the underlying tissue are central key-mechanisms in the generation of an inflammatory response, e.g. in wound healing, infections, etc.. However, many diseases with an inflammatory component are the result of this beneficial recruitment process getting out of control. It is generally considered that atherosclerosis is among the group of chronic diseases, in which recruitment of monocytes and to a lesser extent T–cells is the root of the pathology. The recruitment of monocytes and T–cells requires the expression of various classes of adhesion molecules like selectins, intercellular adhesion molecule 1 (ICAM–1), and vascular adhesion molecule 1 (VCAM–1) as well as the presence of counter receptor molecules on leukocytes and endothelial cells (for review see [71]). Furthermore, a chemotactic gradient to guide monocytes and T–cells to the source of the inflammatory signal is necessary. In atherosclerosis, the inflammatory signal originates in the vessel wall.

Oxidized low density lipoprotein (LDL) located in the sub-endothelial space plays a role in increasing the adherence of monocytes and their migration into the vessel wall. Oxidized LDL upregulates adhesion molecules on endothelial cells [72] and the expression of MCP–1 in endothelial and vascular smooth muscle cells [73]. During oxidation of LDL a large amount of the phosphatidylcholine (PC) is converted to lysoPC through phospholipase A_2 activity. LysoPC has also been

shown to induce the expression of adhesion molecules and MCP–1 on endothelial cells [74]. A non-oxidized, enzymatically degraded form of LDL was found in atherosclerotic plaques. It was demonstrated to cause monocyte adhesion and migration to the sub-endothelium and to induce MCP–1 expression and release from macrophages [75].

Arterial bifurcations, branches and curvatures, which typically characterize lesion prone areas, cause alterations in blood flow patterns in these areas. Interestingly, it has been shown that shear stress upregulates the expression of ICAM–1 [76] and MCP–1 [77].

In human atherosclerotic lesions, MCP–1 has been found to be expressed mainly by macrophages, but also by smooth muscle cells [78]. The MCP–1 protein has also been detected on the luminal endothelium of early human atherosclerotic lesions [79]. It was suggested that MCP–1 has an intimate involvement in the continued infiltration of monocytes into lesions and perhaps other downstream chronic modulatory events.

In a recent *in vitro* study, the effect of MCP–1 and IL–8 on endothelial cell monolayers expressing E-selectin under flow conditions was studied [80]. It was found that MCP–1 and IL–8 could convert monocyte rolling to a firm adhesion, demonstrating its importance for atherosclerotic plaque development and progression.

CCR2 mRNA is decreasing during monocyte differentiation into macrophages. Oxidized LDL reduces CCR2 mRNA of a monocyte cell line, which is probably due to its ability to stimulate monocyte differentiation to macrophages [81]. However, non-modified LDL possesses an opposite activity. LDL increased CCR2 mRNA and protein levels of the monocyte cell line, resulting in increased chemotactic response to MCP–1. The differential regulation of CCR2 suggests that its main function is the recruitment of monocytes from the bloodstream and that perhaps other chemokines/receptors are more important for movement of macrophages within lesions. Downregulation of CCR2 receptors on monocytes upon entering the lesion could serve to maintain the cells in a specific area of the inflammation until other chemokine signals are transmitted.

Macrophages are present throughout all stages of atherosclerosis and are considered to be the major inflammatory mediators during disease progression. After entering the atherosclerotic lesion environment, macrophages function as scavenger cells internalizing modified lipoprotein particles, becoming foam cells [82]. Macrophages are the richest source of chemokines and growth factors in

atherosclerotic regions, indicating that macrophages (monocytes) and, thus, MCP–1 play a key role in atherosclerosis. Additionally, macrophages act as antigen-presenting cells to T–cells during the immune response, which occurs during the disease [83].

The presence of T–cells in atherosclerotic lesions indicates that adaptive immunological events in conjunction with inflammatory ones are implicated in atherogenesis. T–cells along with macrophages occur in large numbers at the sites of plaque rupture. Because T–cells secret inflammatory cytokines, which are capable of inducing metalloproteinase expression by macrophages, they are thought to be involved in the development of unstable plaques [83]. Knowing that MCP–1 is also a chemoattractant for T–cells and, therefore, implicated in the development of unstable plaques, things have come full circle [84].

Vascular smooth muscle cells are also a source of MCP–1 under certain conditions. Balloon injury provokes a transient upregulation of MCP–1 in vascular smooth muscle cells. Cultured rat aortic vascular smooth muscle cells stimulated by platelet-derived growth factor showed a similar increase in MCP–1 expression [85].

Interestingly, the potent vasoconstrictor angiotensin II, playing such an important role in many cardiovascular diseases, can directly upregulate MCP–1 expression in rat vascular smooth muscle cells [86]. Vascular smooth muscle cells express mRNA for the MCP–1 receptor CCR2. However, no conclusive data are available about the role of MCP–1 as a positive or negative regulator of proliferation.

CCR2 knockout mice crossed with ApoE knockout mice demonstrated upon feeding with a Western type diet markedly decreased atherosclerotic lesion formation [87]. Furthermore, MCP–1 knockout mice crossed with LDL receptor knockout mice showed a decreased lesion size and a reduction of macrophages within the lesion [88]. The fact that there are still macrophages in the lesions, albeit fewer, suggests that other chemokines are also playing a role in the disease. On the contrary, mice overexpressing MCP–1 crossed with ApoE knockout mice displayed accelerated atherosclerosis through an increased macrophage number in the lesion and lipid accumulation [89]. These studies provide direct evidence for a direct role of MCP–1 and CCR2 in monocyte recruitment during atherosclerosis. However, these animal models show that interfering with a single chemokine or receptor can have profound effects on disease progression without an effect on plasma lipids.

3.2.3. MCP–1 and Congestive Heart Failure

Serological investigations of patients with congestive heart failure (CHF) have demonstrated elevated circulating levels of MCP–1 [90]. MCP–1 levels correlated significantly inversely with left ventricular ejection fraction and were particularly raised in patients with coronary artery disease, connecting atherosclerosis with CHF. The serum of CHF patients enhanced the generation of superoxide anions of monocytes *in vitro*. This effect could be inhibited by neutralizing antibodies against MCP–1.

Heart failure and cardiac hypertrophy induced by pressure overload in hypertensive rats increased the expression of MCP–1 in the heart significantly [91]. The MCP–1 protein was localized in endothelial cells and interstitial macrophages. The number of interstitial macrophages was increased in these animals. These facts suggest that MCP–1 induced macrophage infiltration plays a role in the development of cardiac hypertrophy and failure.

In another study, CHF was induced by volume overload via an aortocaval fistula [92]. MCP–1 expression correlated with the severity of CHF. The protein was localized in cardiac myocytes, endothelial cells, vascular smooth muscle cells, macrophages, and interstitial fibroblasts, and the intensity increased with the severity of CHF. Interestingly, MCP–1 binding sites to the membrane fraction of the myocardium decreased with the progression of heart failure.

Chronic nitric oxide (NO) inhibition in rats induced MCP–1 expression, vascular inflammatory changes, medial thickening and perivascular fibrosis as a sign of atherosclerotic changes, and cardiac fibrosis [93]. The expression of MCP–1 was especially increased in endothelial cells and monocytes in inflammatory lesions. One of the initial experiments of the study demonstrated the potency of MCP–1 injection into the skin of control rats recruiting monocytes to these areas. Administration of MCP–1 antibodies resulted in an inhibition of monocyte infiltration of rat dermis injected with MCP–1. The experiment furthermore revealed that there is a dose dependent effect of the antibody treatment. Infiltration of polymorphonuclear cells into the dermis induced by IL–8 injection was not affected by the administration of MCP–1 antibodies. Antibody treatment in rats with NO inhibition resulted in inhibition of the medial thickening of large and small coronary arteries but was not able to prevent perivascular fibrosis and cardiac fibrosis. Furthermore, the increase in TGF–β_1 (transforming growth factor β_1) and collagen I expression due to NO inhibition could not be inhibited by application of MCP–1 antibodies. This study demonstrates that even though atherosclerosis and CHF are closely linked by many pathophysiological and

anatomical features, a variety of distinct processes have to be considered. A major role of MCP–1 in these diseases is evident. However, more evidence has to be collected to precisely understand its function and influence on the different disease processes.

3.2.4. MCP–1 and Myocarditis

In 1998, Kolattukudy et al. reported their findings about the cardiac-specific overexpression of MCP–1 in mice [94]. Transgenic mice showed leukocyte infiltration into the interstitium between cardiac myocytes. The infiltrate consisted mainly of macrophages but the presence of a significant amount of T–cells was not observed. At an age of 100 days these mice did not display major fibrosis or vascular alterations. Myocardial samples of transgenic mice analyzed for the expression of IL–1, IL–2, IL–6, TNF–α (tumor necrosis factor α), and TGF–β (transforming growth factor β) mRNA did not show the production of any of these proinflammatory or morphogenic chemokines. This indicates that there was no local leukocyte or endothelial cell activation up to 100 days of age. Echocardiographic analysis of approximately one year old transgenic mice revealed a substantial cardiac hypertrophy and dilation when compared to nontransgenic control mice. Left ventricular mass and both systolic and diastolic left ventricular internal diameters were increased and contractile function was decreased. Heart mass of transgenic mice was increased by approximately 65%. Histological analysis of the myocardium of these mice showed hypertrophy of myocytes, mild interstitial edema, moderate myocarditis and a limited amount of fibrosis. These findings closely resemble the situation in humans with myocarditis. Thus, targeted expression of MCP–1 in the adult heart muscle produces a pathological condition that shares many features associated with myocarditis/cardiomyopathy.

In another study, the contribution of monocytes/macrophages to compensatory neovascularization was evaluated in the same transgenic mouse line with cardiac-specific overexpression of MCP–1. The investigators observed tunnels in the myocardium of these mice that were free of endothelium and occasionally contained blood-derived cells. Their data suggest that monocytes/macrophages drill tunnels by using the broad-spectrum mouse macrophage metalloelastase. Tunnels were characterized by neo-endothelial markers and proliferation markers suggesting an active angiogenic process. However, this study does not

show whether these tunnels eventually become capillaries connected to an existing vessel and colonized by circulating endothelial cell progenitors.

Data available from the literature prove that MCP–1 is a potent chemokine influencing inflammatory events, atherosclerosis, congestive heart failure, and myocarditis. Cardiac-specific overexpression of MCP–1 results in a pathological condition that closely resembles the situation in humans suffering from myocarditis. Preliminary experiments performed in our laboratory demonstrated the development of severe congestive heart failure in these transgenic mice. Exercise tolerance is used as a primary marker for the severity of CHF in humans and the base of the New York Heart Association (NYHA) classification system. In order to evaluate whether these mice demonstrate impaired exercise tolerance and to evaluate the progression of heart failure we conducted experiments to access the exercise capacity of these mice every four weeks starting at an age of 16 weeks.

3.3. Physical Activity - Angiogenesis and Arteriogenesis

3.3.1. Introduction

Physical activity-induced sprouting and formation of new capillaries (angiogenesis) in the exercising muscle has been reported as early as 1934 [95]. In the 1960s, the increased number and/or size of collateral vessels in patients with peripheral arterial obstructive disease (PAOD) due to exercise had been postulated [96-98]. The development of functionally relevant arteries from preexistent collateral arterioles (arteriogenesis) was hypothesized and later confirmed [99].

The induction of angiogenesis and arteriogenesis are considered very important therapeutic goals in the treatment of PAOD. Ever since the initial findings of vascular changes and adaptations to physical activity, great efforts have been made to determine the underlying mechanisms.

Therefore, this chapter is written to:

- Summarize the physiological adaptations of the vasculature to physical activity,
- Explore their underlying mechanisms,
- Suggest new methods for therapeutic use of physical activity in the treatment of PAOD.

3.3.2. Physical Activity and Angiogenesis - Background

Angiogenesis is mainly occurring in pre- and neonatal developmental stages and usually finished at the onset of adulthood. However, numerous studies have demonstrated that angiogenesis is a common adaptation of the adult skeletal muscle in response to exercise (for reviews see [100,101]).

The angiogenic response of the rabbit extensor digitorum longus muscle to low-frequency stimulation could be observed four days after the onset of stimulation [102]. Daily endurance treadmill running increased capillary-to-fiber ratio of the rat gastrocnemius and soleus muscles within one week of training [103].

These studies indicate that angiogenesis is occurring during relatively short stimulus duration. They also demonstrate the increase in capillary-to-fiber ratio for the entire duration of the skeletal muscle stimulation. Nevertheless, upon cessation of the exercise regime, the angiogenic adaptation to increased physical activity is reversed under physiological conditions [104]. It is unknown, whether the exercise-induced increase in capillarity is reversible or persistent under pathophysiological conditions like PAOD.

It is generally believed that angiogenesis is mediated by certain growth factors that act on endothelial and vascular smooth muscle cells. Since the early 1990s it became apparent that these growth factors are released in response to physical activity.

3.3.3. Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is one of the growth factors that received a lot of attention in this context. The homodimeric glycoprotein is known to exist in at least five isoforms. Three different receptors that are restricted to endothelial cells have been characterized [105-107]. In 1995, Hang et al. [108] reported upregulation of VEGF gene expression in skeletal muscle of the rat exposed to electrical stimulation. This observation was confirmed by Breen and coworkers [109], who found increased skeletal muscle VEGF mRNA in rats after a single exercise bout.

In 1999, two independent groups observed increased VEGF mRNA levels after single exercise bouts in human skeletal muscle [110,111]. VEGF expression is known to be stimulated by hypoxia [112,113], which seems to be at least partially mediated by hypoxia inducible factor 1 (HIF–1) [114]. Gustafsson et al. [111] investigated the relationship of VEGF and HIF–1 mRNA in response to exercise. These investigators found that the exercise-induced increase in expression levels of VEGF correlated with the increase in HIF–1α and HIF–1β mRNA. Furthermore, these changes correlated with the increase in femoral venous lactate concentration. However, when blood flow was restricted by 15-20%, no further increase in the expression levels of VEGF, HIF–1α and HIF–1β could be observed. Similarly, in the study performed by Richardson and colleagues [110] skeletal muscle VEGF mRNA increased in response to a single exercise session. But no further increase in the expression of VEGF was found when inhalation of a hypoxic gas during exercise caused an even greater reduction of intracellular

PO_2. The results of these two independent studies suggest that the increase in VEGF mRNA is at least partially mediated by the increase in HIF–1 due to a single bout of exercise. The moderate skeletal muscle hypoxia caused by a single exercise bout seems to be sufficient to induce VEGF expression. It appeared that normoxic exercise achieves the postulated intracellular PO_2 "threshold" for the VEGF response. A further decrease of the intracellular PO_2 either by restriction of blood flow or inhalation of hypoxic gas during exercise does not result in a greater elevation of VEGF expression compared to normoxic exercise.

Interestingly, chronic hypoxia (inspired O_2 fraction of 0.12 for eight weeks) was shown to attenuate resting as well as exercise-induced VEGF mRNA levels in rats [115]. Furthermore, it was demonstrated that mRNA of two VEGF receptors (flt–1 and flk–1) decreased in response to chronic hypoxia under resting conditions and after exercise. These findings could provide an explanation for the lack of increased capillarity due to chronic hypoxia.

In 2000, Richardson et al. [116] revealed that the VEGF response to exercise is attenuated in trained individuals, suggesting a negative feedback. In trained individuals capillary-to-fiber ratio increased as well as maximal skeletal muscle oxygen extraction and oxygen conductance. These findings are in accordance to the current opinion: When structural and functional requirements of the exercising skeletal muscle are met, no further angiogenic response e.g. increased VEGF expression is needed.

An endurance exercise regime of five days in rats was shown to cause an attenuated VEGF response [117]. After the first exercise bout VEGF expression was maximal. After exercise at the second day VEGF expression was still significantly elevated when compared to resting levels but reduced when compared to VEGF mRNA after the first exercise bout.

Recently, Gustafsson et al. [118] reported a twofold increase in VEGF at both the mRNA and the protein levels in heart failure patients that underwent a training regime of eight weeks. The importance of these findings is demonstrated by the study of Duscha and coworkers [119] who showed that capillary density is markedly decreased in heart failure patients. The decrease in skeletal muscle capillarity appears to be a contributing mechanism for the exercise intolerance observed in this patient group. Based on the knowledge that the VEGF response to exercise is intact in heart failure patients the role of physical activity for the maintenance of skeletal muscle capillary density can be appreciated.

Nitric oxide (NO) is released during exercise, induces VEGF expression *in vitro* [120], and is known to be implicated in the signaling pathway of VEGF-induced angiogenesis [121]. Benoit et al. [122] investigated the potency of NO application for one hour to induce VEGF expression *in vivo*. They observed an increase in VEGF mRNA upon application of NO and suggested that the release of the vasodilator NO could be involved in exercise-induced angiogenesis. However, the magnitude of the NO-induced VEGF expression does not achieve the exercise-induced VEGF mRNA level.

Inhibition of the nitric oxide synthase was demonstrated to attenuate the VEGF response to a single exercise bout in rats [123]. This is again suggesting that exercise induced NO release plays an important role in the angiogenic adaptation of the skeletal muscle by either inducing VEGF transcription or increasing VEGF stability.

3.3.4 FGF–1 and FGF–2

The fibroblast growth factor (FGF) family and the mediating receptors have extensively been studied (for reviews see [124-126]). FGF–1 and FGF–2 were shown to induce angiogenesis *in vitro* and *in vivo* [127,128].

In contrast to VEGF expression, the response of FGF–1 and/or FGF–2 expression to physical activity remains to be determined. It was found that chronic electrical stimulation of rat skeletal muscle for five days leads to elevated mRNA levels for FGF–1, FGF–2, FGF receptor 1 (FGFR1), and FGF receptor 4 (FGFR4) [129]. After longer stimulation FGF–1 and FGFR4 mRNAs decreased to normal levels, whereas FGFR1 mRNA returned to normal levels after ten days but increased again after 20 days of stimulation. FGF–2 expression remained elevated over the whole experimental period. When satellite cell cultures were stimulated FGF–1 and FGF–2 expressions also increased but to a lesser extend in comparison to intact skeletal muscle. FGF–1 and FGF–2 proteins increased in electrically stimulated rabbit skeletal muscle. These changes were concomitant with increased mitotic activity, fast to slow skeletal muscle fiber conversion and increased capillarity [130].

A short-term exercise program of rats did not cause increased FGF–2 expression [117] when compared to resting levels. A single bout of submaximal exercise also didn't lead to a response of FGF–2 expression [123]. There was no FGF–2 response to a single exercise bout in rats under normoxic conditions or

after a chronic hypoxia period of eight weeks [115]. However, it was shown earlier by the same group that the same exercise stimulus leads to increased FGF–2 expression in the rat [109]. In humans, these investigators reported no measurable FGF–2 expression after a single endurance exercise bout [110]. Similarly, Gustafson and coworkers did not observe increased FGF–2 mRNA after a single exercise bout in the human [111].

A short-term exercise program in rats with bilateral femoral artery occlusion led to increased capillarity in the gastrocnemius muscle when compared to sedentary control rats [131]. The increase in capillarity was caused by endothelial cell proliferation. However, FGF–2 content of the gastrocnemius muscle was not altered by exercise, suggesting that other endothelial cell mitogens are responsible for the observed changes.

Overload of a particular skeletal muscle by removal of the synergist has been reported to cause increased capillary-to-fiber ratio [132]. This change was not associated by an increase in FGF–2 expression. No FGF–2 immunoreactivity was observed in capillaries, whereas it was present in larger vessels and nerves. There was no difference in maximal muscle blood flow between overloaded muscle and muscle of unoperated control rats, suggesting that neither FGF–2 nor increased blood flow are responsible for the increase in capillarity.

The role of FGFs in exercise induced angiogenesis remains to be clarified. Electrical stimulation of rat skeletal muscle appears to induce expression of FGF–1 and FGF–2 mRNAs and to increase their protein levels. However, most of the endurance exercise studies in both rats and humans did not lead to a significant response of FGF expression. It has to be mentioned that most of these investigations concentrated on the expression levels while paying no attention to the protein levels.

It has been questioned whether FGF–1 and FGF–2 are secreted via the endoplasmic reticulum since they lack the conventional leader sequences. Cellular damage and stretching as well as a contraction based mechanism have been suggested to cause the release of FGFs from cells *in vivo* [133-137]. Thus, a possible influence of physical activity on the release of intracellular FGFs can not be excluded and further investigations are needed.

Recently, Maciag and colleagues started to resolve the puzzle of FGF–1 release. The release of FGF–1 may proceed through a novel release/export pathway. They found that FGF–1, but not FGF–2, is released as a latent homodimer by a transcription- and translation-dependent mechanism in response to a variety of

cellular stresses including heat shock [138], hypoxia [139], and serum starvation [140]. The disruption of communication between the endoplasmic reticulum and Golgi apparatus by brefeldin A does not prevent the release of FGF–1 from 3T3 cells [138], which confirms that FGF–1 release may occur through a nonconventional pathway. FGF–1 is released *in vitro* complex sensitive to reducing agents and denaturants. The complex contains the p40 extravesicular domain of the calcium-binding protein p65 synaptotagmin 1 [141]. The expression of synaptotagmin 1 is essential for the release of FGF–1 in response to stress [142]. Furthermore, it was demonstrated that FGF–1 isolated from tissues as a high molecular weight aggregate exists as a component of a noncovalent heparin-binding complex with p40 synaptotagmin 1 and S100A13, a member of the S100 gene family of calcium-binding proteins [143]. The precursor form of interleukin 1α was shown to block the release of FGF–1 suggesting that their release pathways may be mechanistically linked [144]. The anti-allergic drug amlexanox, which binds S100A13, is able to inhibit the release of FGF–1 and p40 synaptotagmin 1 in response to temperature stress [145]. Amlexanox also causes the reversible disassembly of actin stress fibers indicating that the actin cytoskeleton also plays a role in the regulation of FGF–1 release. The expression of S100A13 facilitates the release of FGF–1 into the extracellular compartment in response to temperature stress *in vitro* [146]. Interestingly, the expression of S100A13 was demonstrated to reverse the sensitivity of FGF–1 release to inhibitors of transcription and translation [146]. S100A13 may also induce the formation of a noncovalent FGF–1 homodimer, which is essential for FGF–1 release [146]. The most recent study of Maciag and colleagues showed that copper induces the formation of a multiprotein aggregate between S100A13, FGF–1, and p40 synaptotagmin 1 [147]. When copper was bound by a copper chelator, the heat shock-induced release of FGF–1 and S100A13 was repressed in a dose-dependent manner. However, the mechanism by which the multiprotein complex is finally released to the extracellular space remains to be determined. Maciag and colleagues anticipate that phosphatidylserine flipping from the inner leaflet to the outer leaflet of the plasma membrane might be involved in this mechanism since S100A13, FGF–1, and p40 synaptotagmin 1 are phosphatidylserine-binding proteins [146].

Since FGF receptors are not restricted to endothelial cells and biological effects of FGFs on other cell types have been shown, skeletal muscle adaptations in response to physical activity beyond angiogenesis remain to be determined.

3.3.5. Other Growth Factors

The angiogenic effect of transforming growth factor–β_1 (TGF–β_1) is not directly mediated (for review see [148]). However, TGF–β_1 is essential in the process of vasculogenesis [149,150]. A shear stress response element located in the TGF–β_1 promoter region causes increased TGF–β_1 expression of vascular endothelial cells upon submission to increased shear stress *in vitro* and *in vivo* [151,152]. A single exercise bout as well as hypoxia at rest increased TGF–β_1 expression in rats [109]. However, exercise under hypoxic conditions did not further increase TGF–β_1 mRNA. To precisely determine the role of TGF–β_1 expression in response to exercise further studies on different species are needed. The long-term effects of exercise on TGF–β_1 expression also with regard to shear stress-induced regulation remain to be determined.

Platelet derived growth factor (PDGF) expression is induced through shear stress via two shear stress response elements in its promoter region [153-155]. PDGF is similar to TGF–β_1 essential for vasculogenesis [156]. However, no studies have been performed investigating the effect of exercise on PDGF expression even though increased mRNA levels could be expected via greater shear stress due to exercise.

Endothelial cell-stimulating angiogenic growth factor (ESAF) has been shown to be implicated in angiogenesis possibly via regulation of enzymes involved in the angiogenic process like procollagenase, prostromelysin 1, progelatinase A, and tissue inhibitor of metalloproteinase 1 (TIMP–1) [157,158]. Brown et al. [159] reported that chronic electrical stimulation of rat skeletal muscle leads to increased activity of ESAF. The same was demonstrated to occur in overloaded skeletal muscle of the rat [132].

Even though a close relationship between angiopoietins and VEGF has been observed and their importance in the process of blood vessel formation has been demonstrated [160,161] no studies have been published regarding the influence of physical activity on this new group of growth factors.

3.3.6. Physical Activity and Arteriogenesis - Background

One of the first studies that explored the role of exercise for the treatment of PAOD was done by Foley in 1957 [162]. He reported faster healing of gangrenous feet when patients were subjected to a walking program. The German clinician

Schoop focused mainly on the importance of the collateral circulation [163]. He hypothesized that the speed of blood flow, but not ischemia, causes an increase in the collateral circulation and that exercise is the simplest, most effective method for augmenting the collateral circulation. He even suggested a precise interval training program for the treatment of PAOD. Unfortunately, these studies were mainly based on clinical impressions and little data were presented to prove the hypothesis and the effect of exercise on PAOD in general and on the collateral circulation in particular. In 1967, Skinner and Strandness [96] investigated the effect of a walking exercise program on three different parameters in patients with PAOD. Systolic pressure at the ankle after exercise, maximal walking time, and the time of the onset of claudication pain were significantly increased in all of the five study subjects. They concluded, without supplying further data, that a significant increase in collateral circulation of long-term exercised patients is improving the blood supply to the obstructed leg. In 1969, Alpert et al. [98] observed a significant correlation between the improvement in maximal walking time and the increase in calf blood flow during exercise after a six months walking regime. Based on their results, the authors concluded that functional factors (e.g. better coordination of the working muscles) as well as anatomic factors (increase in the number and/or size of collateral vessels) are involved in the improved performance of patients after regular physical activity.

In 1968, Sanne and Sivertsson [97] performed one of the first true experimental studies with regard to exercise and PAOD. After unilateral ligation of the femoral artery cats were trained for five weeks on a treadmill. The collateral resistance at maximal vasodilation was shown to be significantly reduced in trained cats, when compared to sedentary controls. They also reported that the flow resistance in the distal vascular bed was not changed after occlusion and not affected by exercise. Even though not providing any histological evidence they suggested that the “spontaneous” growth of the collateral vessels in the occluded limb was very good. Five weeks after occlusion, the collateral resistance during maximal vasodilation decreased almost exactly threefold in the untrained animals. The authors hypothesized that the “normal” stimuli for collateral development, whatever their nature would be, are very efficient. They concluded that physical exercise is a very potent additional stimulus, which further enhances the development of collateral vessels. This study, being very elegant for its time, was pointing into a new direction. From there on, more attention was paid to collateral vessel development as a therapeutic mechanism for the treatment of PAOD.

3.3.7. Development of Collateral Arteries

The terms “collateral circulation” and “collateral resistance” were used for decades. Their changes due to different treatments, interventions and physiological processes have been detected without precisely understanding the underlying mechanisms. In 1971, Schaper et al. [99], who was mainly focusing on coronary collaterals at the time, provided evidence that the fall in collateral resistance was caused by active growth of preformed arterioles. A new paradigm was formed: Collateral arterioles grow by mitotic cell division after DNA replication of endothelial and smooth muscle cells. Recently the term “arteriogenesis” has been ascribed to the process of collateral artery growth [164]. Arteriogenesis is now considered the only relevant type of vascular growth that potentially saves limbs, hearts, and brains. Angiogenesis, the growth of capillaries, can never replace a major occluded artery. Therefore, enhancement of arteriogenesis is becoming the main target for researchers and physicians involved in the treatment of PAOD. Arteriogenesis has been shown to take place in hindlimbs with femoral artery occlusion [40,165,166]. This process is restricted to the thigh, whereas angiogenesis is predominantly occurring in the calf muscle and is associated with hypoxia. The regulating mechanisms for arteriogenesis are not yet entirely clarified. However, the results of several studies suggest that the arterial diameter increases when wall shear stress is elevated [167-171]. This process continues until wall shear stress has normalized. It has been demonstrated that shear stress induces expression and release of FGF–2 from vascular smooth muscle cells and endothelial cells [172,173]. Increased shear stress was also shown to induce expression of MCP–1 [174] and the upregulation of cell adhesion molecules in the proliferating arterioles [39]. The adhesion of monocytes and their subsequent transformation to macrophages seems to be essential for arteriogenesis. Macrophages release a variety of growth factors and cytokines known to be important for arterial growth e.g. FGF–2, MCP–1, TNF-α, and MMPs (matrix metalloproteinases). (see also chapter 3.2.)

3.3.8. Exercise-Induced Arteriogenesis - The Rat Model

Numerous studies demonstrated the potential role of exercise in the induction of arteriogenesis in many species. However, both positive and negative results have been reported.

The rat model of PAOD and exercise as a stimulus for arteriogenesis was mainly established by Terjung and coworkers. In 1986, the results of their first study about the influence of exercise on rat hindlimbs with femoral artery occlusion were reported [175]. Their results indicate a clear beneficial effect of exercise on maximal running duration and intensity. Functional performance of the gastrocnemius-plantaris-soleus muscles in trained occluded animals was similar to unoperated control rats but reduced in sedentary occluded animals. However, the blood flow determination of this muscle group by microspheres did not yet provide conclusive results about the exercise-induced arteriogenesis with subsequent increase in skeletal muscle perfusion during exercise.

Four years later this work was continued with slightly modified techniques [176]. After an endurance exercise program of six weeks no significant difference in total hindlimb blood flow was observed between acutely occluded, sedentary occluded, and trained occluded animals. Total hindlimb blood flow reached only 46-63% when compared to unoperated control rats. However, a significant difference could be observed when blood flow distribution was analyzed. Acutely occluded animals had a markedly reduced blood flow to the gastrocnemius-plantaris-soleus muscle group when expressed relative to the proximal blood flow. In sedentary occluded animals, distal blood flow partially recovered and further increased in trained occluded animals. The authors suggested that the increase in blood flow to the distal hindlimb in trained animals could be due to better collateral vessel function. However, the total hindlimb perfusion could not be recovered by treatment with an endurance exercise program for six weeks.

In the same year, Terjung and colleagues could detect a significant increase in total hindlimb blood flow in rats with femoral artery occlusions trained for up to eight weeks [177]. Again a significantly greater redistribution of blood flow to the gastrocnemius-plantaris-soleus muscles was observed in trained occluded rats when compared to sedentary occluded rats.

In 1993, the same group reported increased blood flow to the total hindlimb, to proximal muscles, and to distal muscles, when the femoral artery was occluded after pretreatment with an ACE inhibitor for up to seven days [178]. The authors hypothesized that a larger diameter of the collateral vessels due to ACE inhibition could be responsible for the increased blood flow. In a follow-up study [179], it was observed that chronic administration of an ACE inhibitor combined with physical activity for three weeks elevated total hindlimb blood flow and exercise tolerance to a greater extent than physical activity or ACE inhibition alone.

The next pharmacological intervention applied to the same model was chronic heparin treatment [180]. It was demonstrated that heparin treatment further enhanced blood flow to the total hindlimb, to proximal muscles, and to distal muscles. Heparin treatment in combination with physical activity also increased contractile force of the gastrocnemius-plantaris-soleus muscle group as well as exercise capacity when compared to sedentary heparin treated animals. This study was particularly interesting because heparin is known to interact with angiogenic growth factors. Through its high-affinity binding, heparin aids in the storage and possibly in the mobilization of growth factors in and from the extracellular matrix (for reviews see [181-183]). It has been shown to interact with cell surface receptors and growth factors initiating the growth factor signaling. Therefore, it is not surprising that heparin enhances the process of collateral artery growth by possibly potentiating the effects of growth factors released due to physical activity.

In 1996, these investigators determined the influence of FGF–2 on collateral artery dependent blood flow [184]. FGF–2 infusion via osmotic pumps for four weeks markedly increased blood flow to the total hindlimb, to proximal muscles, and to distal muscles. Muscle performance of the gastrocnemius-plantaris-soleus muscle group was enhanced and capillary-to-fiber ratio of FGF–2 infused rats was increased in high-oxidative fiber sections but not in a predominantly low-oxidative section when compared to acutely ligated rats and to heparin infused control animals. Postmortem angiograms revealed more collateral arteries and a better filling of the femoral artery distal to the point of occlusion. Thus, the authors proved FGF–2 treatment to be very efficient in induction of arteriogenesis upon femoral artery ligation.

When the stimulating effect of FGF–2 was combined with physical activity, a more pronounced increase in collateral artery dependent blood flow could be observed [185]. Hindlimb blood flow measurements compared to the sedentary carrier group revealed an increase in the following order: trained carrier group, sedentary FGF–2 group, trained FGF–2 group. Interestingly, blood flow to the proximal hindlimb muscles was especially enhanced in the trained FGF–2 group indicating a significant treatment interaction. However, when muscle performance was analyzed the two sedentary groups could not maintain the tension as well as the trained carrier group. Again, the interaction of training and FGF–2 application led to the highest increase e.g. muscle performance. These data indicate that muscle performance is not just determined by blood flow to the working muscle. Therefore, other factors must have contributed to the enhanced muscle performance of the trained rats. Unfortunately, the

investigators did not determine blood flow and muscle performance in unoperated control animals. It would be very interesting to compare the potency of the combined treatment with the physiological conditions. This could reveal whether blood flow to the occluded limb is partially or even totally restored and thus, the true importance of the combined treatment. Additionally, capillary-to-fiber ratio increased only in the low-oxidative gastrocnemius section of trained rats opposing their findings of the previous FGF–2 infusion study [184].

In 2000, the efficacy and specificity of FGF–2 application on the increase in collateral artery blood flow were determined [186]. The investigators found similar increases in the intra arterially infused group when compared to the intra venously infused group or the subcutaneously injected group as long as similar amounts were administered. There was no obvious difference in collateral artery blood flow when FGF–2 application was short-term or prolonged and continuous or intermittent. Chronic FGF–2 treatment did not alter blood flow upon acute femoral artery occlusion when compared to untreated or carrier treated control rats. Therefore, the authors conclude that FGF–2-induced arteriogenesis requires vascular occlusion and can be restricted to short-term application though different routes to be effective.

In the same year, these investigators evaluated the influence of an endurance exercise program before acute occlusion of the femoral artery on collateral artery blood flow [187]. Prior endurance exercise training increased blood flow to the hindlimb in the distal as well as in the proximal segment when compared to sedentary control rats. Blood flow to the gastrocnemius-plantaris-soleus muscles was significantly increased but did not nearly reach the level of trained FGF–2 infused rats that was shown earlier [185]. These results indicate that prior exercise training is effective in increasing blood flow to the distal hindlimb muscles upon acute occlusion of the femoral artery. But since there is no need for an excessive use of the preexistent collateral arterioles during exercise with intact legs the increase in shear stress is just enough to induce a moderate growth of these vessels.

The most recently published study by Terjung and colleagues indicates that nitric oxide (NO) is implicated in arteriogenesis induced by FGF–2 as well as VEGF [188]. Nitric oxide synthase (NOS) inhibition abolished the effect of both FGF–2 and VEGF on arteriogenesis. Both growth factors alone were capable of increasing blood flow to the total hindlimb, to proximal muscles, and to distal muscles to a similar extent when compared to untreated control rats. However, NOS inhibition together with FGF–2 or VEGF treatment resulted in unchanged

blood flow when compared to untreated control rats. Thus, reduced NO production, availability, and/or responsiveness that are frequent phenomenon in patients with PAOD and CHF [189-191], could inhibit the arteriogenic response to FGF–2 and VEGF treatments.

Summarizing the results of Terjung and colleagues, one could speculate that the ideal treatment for rats with an arterial occlusion would begin even before the onset of occlusion with at least six weeks of treadmill running. After femoral artery occlusion FGF–2 application via different routes has proven to be extremely effective when combined with an endurance regime for at least four weeks. In this model, exercise appears to multiply the effect of FGF–2 via further increase in shear stress and increased release of NO (for review see [192]). It has also been shown that FGF–2 application to vascular smooth muscle cells *in vitro* upregulates the expression of VEGF [193]. A marked synergist effect on VEGF expression could be observed when hypoxia was combined with FGF–2 application. This study suggests that FGF–2 and skeletal muscle hypoxia during exercise could have promoted among other factors an amplified VEGF response in trained FGF–2 rats.

However, Terjung and colleagues did not show that a treatment exists, which fully restores the dilatory capacity of the normal vasculature in a hindlimb with femoral artery occlusion. It would be very interesting to investigate the level of blood flow recovery to the total hindlimb that can be achieved with the “optimal” therapy. Nevertheless, the optimum remains to be determined. There could be significant additive effects of a combined VEGF and FGF–2 treatment, which could be used even during the initial training period.

3.3.9. The Dog Model of Exercise-Induced Arteriogenesis

In the late 1970s, researchers and clinicians paid much more attention to the possible effect of exercise on the development of the collateral circulation upon occlusion of a major coronary artery. Arteriosclerosis had become the leading cause of death some years ago and cardiovascular medicine was looking for ways to increase perfusion in areas distal to the site of occlusion. The dog model had been shown to be useful for clinicians, surgeons, and researchers at the time for three reasons:

- Surgical techniques used for humans could be applied with slight modifications.

- Dogs develop impressive coronary collaterals that could be visualized by angiography.
- Last but not least dogs proved to be very good runners.

In 1978, Heaton et al. [194] reported that exercise enhanced blood flow only to the endocardium of the collateral dependent zone in dogs with occlusions of the left circumflex coronary artery and of the distal part of the left anterior descending artery. The enhanced endocardial blood flow could only be observed during exercise. The training program of six weeks was sufficient to induce a decrease in heart rate at a given workload. In sedentary control animals blood flow did not change over the same period, which would indicate that no significant arteriogenic response occurred. However, the baseline measurements were performed two weeks after the initial operation, which is the period were the main arteriogenic adaptation occurs. Thus, these results are not indicative for the entire process of arteriogenesis.

Neill and Oxendine [195] reported that trained dogs with occlusion of the left circumflex coronary artery had a higher retrograde flow from the distal part of the occluded artery when compared to their sedentary controls. However, the retrograde flow method does not indicate the true tissue perfusion by collaterals. The greater pressure drop across the coronary collaterals observed in sedentary dogs is most likely due to increased scar formation in the collateral dependent zone of the myocardium. There was no apparent difference in the angiographic images of trained and sedentary dogs. Furthermore, the microsphere technique demonstrated that there was no beneficial effect of physical activity on blood flow to the collateral dependent zone of the myocardium.

In 1981 [196], it was reported that an exercise program initiated three months after occlusion of the left circumflex coronary artery promotes collateral dependent blood flow. Again, the retrograde perfusion method used in this study does not allow conclusions about the tissue perfusion provided by collateral arteries. Daily low intensity exercise for six weeks did not alter collateral dependent blood flow in control dogs with normal coronary arteries.

In 1982, our group at the Max-Planck-Institute in Bad Nauheim conducted a study to precisely determine the role of exercise in the induction of collateral coronary growth [197]. Therefore, we used the rigidly standardized isolated heart model of testing regional blood flows with tracer microspheres during maximal vasodilation and under a wide range of perfusion pressures (40-140mmHg). The dogs performed a vigorous exercise program (up to three month) before the left

circumflex and the right coronary arteries were slowly occluded with ameroid constrictors (one-vessel occlusion was used in most of the aforementioned studies). After a recovery period of two weeks the dogs continued the exercise program for another 12 weeks. The workloads used in this study were the highest reported so far. At the end of the training period the dogs achieved a very high level of physical fitness. They were able to run up an inclination of 22% and a speed of eight miles per hour for one hour. However, there was neither a difference in coronary nor in collateral blood flow in exercised dogs when compared with the sedentary control animals. Collateral conductance in trained and sedentary dogs reached only about 40% of that of the replaced coronary artery. Calculated total collateral resistance of trained dogs was not different from sedentary animals. No significant change in mortality was observed. This study was mainly designed to precisely determine the influence of physical activity on coronary collateral arteries of the dog. The isolated heart preparation allowed the use of fixed pressures under maximal vasodilation and the determination of the corresponding blood flow via use of radiolabled microspheres. The study demonstrated that a very high level of physical activity does not produce an increase in coronary collateral blood flow in dogs and thus, did not provide a further stimulus for arteriogenesis. Because of the advanced techniques used in our study the observations are at variance with other studies that reported beneficial effects of physical activity on collateral dependent blood flow. Nevertheless, we can not exclude the possibility that treadmill running at a certain workload could have produced differences in collateral blood flow when comparing the highly trained dogs and their sedentary controls. Note that the beneficial effect of exercise on final infarct size that was previously reported by us [198] was not again assessed in this study.

A study that partially confirmed this assumption was conducted in the same year [199]. Dogs with a stenosed the left circumflex coronary artery were trained for 12 weeks on a treadmill using sprints and endurance running. When compared to sedentary controls trained dogs exhibited less evidence of left ventricular failure (higher cardiac output, lower left atrial pressure) upon total occlusion of the left circumflex coronary artery during a single exercise session. Trained dogs showed improved collateral dependent blood flow at rest and during exercise while there was virtually no improvement in the sedentary control dogs. The authors concluded that running exercise stimulates the development of collateral vessels in dogs with a stenosed coronary artery, and that the enhanced collateral flow has salutary effects on myocardial function. However, with our findings of myocardial blood flow in mind, these results could also be interpreted

the other way around. Physical activity induced beneficial changes of myocardial function during exercise, leading to enhanced perfusion of the myocardium upon total occlusion during a given level of exercise.

In 1985, Cohen and Steingart reported their findings on coronary collateral development after occlusion of the left circumflex coronary artery in dogs submitted to an endurance exercise program for six weeks [200]. Using thallium–201 scintigraphy during exercise, the investigators revealed a significant improvement in scintigraphic measurements over the first four weeks. Microsphere measurements showed a good recovery of perfusion distal to the site of occlusion. Serial angiographic studies demonstrated the development of collateral vessels and the increase in collateral circulation to the left circumflex coronary artery. Cardiac output during exercise increased and the left atrial pressure decreased significantly during the study period. However, the lack of a real control group makes it difficult to ascribe these beneficial effects to the exercise regime used in this study. Nevertheless, this study demonstrated that collateral coronary artery development is a dynamic process and that exercise hemodynamic measurements improve with progression of arteriogenesis.

Two years later, the same model was used to investigate the effect of an exercise program prior to occlusion of the left circumflex coronary artery on the degree of infarction and collateral dependent blood flow [201]. They observed no significant change in infarct size. The effect of exercise on collateral dependent blood flow did not reach the level of significance. Evaluating the parameters used in this study, no clear beneficial role of prior exercise in cardioprotection was observed.

Cohen and coworkers [202] investigated the role of exercise training in collateral vessel development in dogs with normal coronary arteries. Ten to 12 weeks of endurance exercise resulted in significant adaptations of the cardiovascular system and skeletal muscle. However, the increase in collateral dependent blood flow was not significant, suggesting that exercise is not an appropriate stimulus for significant arteriogenesis in dogs with normal coronary arteries.

In 1990, it was reported that endurance training does not induce changes in collateral development in dogs with normal coronary arteries [203]. After an endurance exercise regime of 12 weeks collateral blood flow determined by microspheres was not different from sedentary animals. The author concluded that exercise in dogs with normal coronary arteries does not alter the development of collateral vessels. This suggests that arteriogenesis is not an adaptation mechanism to an exercise program of 12 weeks in dogs with normal coronary arteries.

Weiss et al. [204] investigated the effect of treadmill walking for over one year on collateral dependent blood flow in dogs with unilateral occlusions of the femoral artery and its branches. At the end of the exercise period blood flow during resting conditions as well as immediately after exercise was determined by microspheres. Interestingly, there was no difference in blood flow between the normal control leg and the occluded leg under both conditions, suggesting that walking exercise induced an immense increase in collateral dependent blood flow to hindlimb muscles. However, due to the lack of a sedentary control group no definite conclusion can be drawn about the true role of physical activity in this study.

In summary, dogs with occlusion of one or two coronary arteries develop large and well visible collateral arteries. The collateral conductance reaches only about 40% of that of the replaced coronary artery leaving room for therapeutic intervention. Exercise has failed to induce arteriogenesis. Increases in tissue perfusion in exercised dogs have mainly been observed during exercise but not under pressure controlled *ex vivo* conditions. Cardiac output increases, heart rate and atrial filling pressure decrease at a given workload. Therefore, regular physical activity could lead to either increased myocardial perfusion at a certain workload due to the increased cardiac output and/or to a reduced myocardial oxygen demand at this workload due to enhanced oxidative metabolism. These questions remain to be solved.

Furthermore, there are no studies that undoubtedly prove the acceleration of arteriogenesis due to exercise in dogs with femoral artery occlusion. However, regular physical activity causes beneficial changes of hemodynamic parameters. Applying these results to the human should not result in questioning the importance of physical activity. Even without paying attention to the anatomical and physiological species differences it should be encouraging for us that trained dogs with coronary occlusions can develop an impressive physical fitness even though their collateral conductance has not changed when compared to sedentary controls.

3.3.10. The Pig Model of Exercise-Induced Arteriogenesis

Bloor and coworkers made major contributions considering the development of coronary arteries due to exercise. In 1984, Bloor et al. [205] investigated the effects of exercise on coronary collateral artery development in pigs with occlusion of the

left circumflex coronary artery. The investigators have chosen the pig as a study species since it has sparse innate coronary collaterals like in humans. Two weeks after placing an ameroid constrictor around the left circumflex coronary artery the pigs were assigned to an endurance exercise regime for five months. Physical activity resulted in an increase of exercise capacity, a reduction of infarct size, and an increase in collateral blood flow to the noninfarcted jeopardized myocardium when compared to sedentary control animals. Therefore, with respect to the utilized exercise regime and the experimental protocol, it appears that collateral vessels developed primarily in or near the ischemic zone of the myocardium and served in tissue salvage especially in the exercised pigs.

The influence of strenuous exercise on cardiac hypertrophy as well as capillary and arteriolar density of pig myocardium was accessed in a later study [206]. A reduction in capillary density and an increase in arteriolar density were reported. However, densities were accessed in number per mm^2 and since myocyte cross sectional area was shown to be increased by 21% in the trained group these data are not conclusive. Nevertheless, the increase in arteriolar density would then be even more impressive when expressed as arteriole-to-fiber ratio. Blood flow was not significantly different at rest, during exercise, and during exercise under maximal vasodilation. The only change in blood flow that could be detected in trained pigs was an increase in epicardial blood flow during exercise and exercise under maximal vasodilation.

In 1987, these investigators reported similar results considering capillary and arteriolar densities [207]. Additional measurements revealed increased maximal oxygen consumption, and increased stroke volume and end-diastolic volume during exercise. The initial reduction in left ventricular end-diastolic diameter during progressive exercise was abolished after the strenuous training regime. This study is again highlighting the importance of functional adaptations of the myocardium to exercise stimuli. Nevertheless, arteriolar growth has been demonstrated to be induced by strenuous physical activity in pig myocardium.

Four years later, another study was carried out to determine the effect of exercise on myocardial blood flow and function after occlusion of the left circumflex coronary artery [208]. An increase in systolic wall thickening during exercise could be observed after a running regime of 25 days. The effect of the training regime on myocardial blood flow was best visible during severe exercise. Subepicardial, submyocardial, and subendocardial blood flow ratios increased significantly when compared to the initial blood flow measurements prior training. Neither systolic

wall thickening or blood flow measurements during exercise changed in sedentary control animals over the study period. The authors concluded that the utilized exercise regime improved myocardial function and blood flow to the collateral dependent myocardium during severe exercise.

In summary, pigs with occlusion of the left circumflex coronary artery respond to regular physical activity with improved myocardial performance. Blood flow to the jeopardized myocardium is increased at a given workload suggesting that arteriogenesis has taken place. Nevertheless, these results are in need of confirmation by other techniques. The isolated heart preparation could be useful to detect if the increase in blood flow during exercise is induced by growth of collaterals or by enhanced myocardial performance. However, exercise has been demonstrated to induce the growth of arterioles in hypertrophied myocardium of pigs. This growth appears to be only one mechanism for blood flow maintenance.

There are no studies available investigating the effect of exercise on arteriogenesis after femoral artery occlusion in the pig.

3.3.11. The Mouse Model of Exercise-Induced Arteriogenesis

To the best of our knowledge, there are no studies considering the effect of exercise on arteriogenesis in mice. In the last years it has been a major issue for our department to develop a mouse model of PAOD. This was especially important since the availability of different transgenic mice give us the possibility to explore the factors increasing and decreasing arteriogenesis. Transgenic animals enable us to explore the underlying mechanisms of arteriogenesis. The small size of the animal allows the use of very expensive and rare compounds. After investing a lot of time and effort, we were able to develop a mouse model of PAOD together with the techniques to detect changes in collateral dependent blood flow via laser doppler imaging (LDI) [209] and magnetic resonance imaging (MRI). We adopted our histological methods to the mouse model to visualize arteriogenesis in the tissue. Angiography of the hindlimbs depicts the growth pattern of the collateral vessels. Determination of exercise capacity provides us with information about functional relevance of the arteriogenesis [210]. We developed a progressive, moderate intensity exercise program for mice with femoral artery ligation to determine the influence of physical activity on arteriogenesis in mice.

The first study conducted in our laboratory concentrated on the effects of general overexpression of FGF–2 in addition to endurance exercise on arteriogenesis. The results of this study are described and discussed in chapters 5.3. and 6.3., respectively.

3.3.12. Exercise and Arteriogenesis in Humans

There are numerous studies that demonstrate the beneficial effect of exercise on increases in both pain free walking distance and maximal walking distance in patients with PAOD (for reviews see [211,212]). However, results are conflicting considering increased blood flow due to regular physical activity in patients with PAOD. There are some studies that report increased blood flow [96,98,213-215], some studies that demonstrate unaltered perfusion [216-221], and one study even reports a decreased resting perfusion [222].

Two recently published studies could be taken as examples for the existing diversity. Gardner et al. [223] reported that calf blood flow was increased in elderly patients with PAOD after taking part in a six-month treadmill exercise program. Blood flow was slightly elevated under resting conditions and more pronounced under reactive hyperemic conditions. The increase in reactive hyperemic blood flow correlated with the increase in pain free walking distance. On the other hand, Tan et al. [224] reported that three month of exercise training did not result in an increase in common femoral artery blood flow.

Explanations for the opposite findings might involve methodological differences in accessing blood flow. There is no “gold standard” that has been established previously and is now used in all of the studies. Furthermore, blood flow is determined at various time points and under different conditions. The length and the intensity of the exercise regime differ markedly. There is no consensus whether blood flow should be accessed under resting conditions, during or immediately after exercise, or during reactive hyperemia. These studies were conducted over a period of almost 40 years, which might explain the use of different methods. Nevertheless, establishing a widely accepted standard for measuring blood pressure in the human could solve this problem for further studies.

It has to be mentioned that exercise proved to exert beneficial effects on endothelial function of patients heart failure [225]. Therefore, the beneficial effect

of exercise training on blood flow could also be ascribed to enhanced endothelial function in some of the experimental settings.

Furthermore, exercise influences a variety of other factors that are likely to contribute to the increase in maximal walking distance. These factors include skeletal muscle metabolism and morphology, blood rheology, regression of atherosclerosis, walking economy, pain perception, and cardiac adaptations (for review see [226]).

Human studies on PAOD often lack histological evidence that exercise induced arteriogenesis. While the induction of angiogenesis can be detected by using muscle biopsies only postmortem tissue preparations could provide the histological evidence for arteriogenesis. Angiography is another tool to determine collateral artery growth but was unfortunately very rarely utilized by most of the studies.

Growth factors therapies for cardiovascular diseases are presently evaluated in clinical studies mainly focusing on coronary collateral development. However, many problems and unresolved issues appear as these trials proceed (for review see [227]). A definite answer on which growth factor is effective in the induction of arterial growth in different cardiovascular diseases can not undoubtedly be given.

There is no study investigating the combined effect of regular physical activity and growth factor treatment in humans.

In conclusion, there is no definite evidence for induction of arteriogenesis in patients with PAOD. However, exercise has been proven to be beneficial for maximal walking distance and quality of life of these patients. Further studies are needed to investigate the role of exercise for arteriogenesis in humans suffering from PAOD.

3.3.13. Summary

That exercise induces angiogenesis has been demonstrated for almost 70 years. The reason for this phenomenon has been discovered during the last years. Expression of various angiogenic growth factors in response to a single exercise bout or to electrical stimulation was demonstrated in different animal models as well as in humans. Among these were VEGF, TGF–β, FGF–1 and FGF–2 including their receptors, and ESAF. However, other potent growth factors like

PDGF and angiopoietins have not been investigated. Angiogenesis is an important adaptive mechanism to increased physical activity. It can be appreciated that the more capillaries are surrounding a skeletal muscle fiber the better is its supply with oxygen, glucose, and free fatty acids. The removal lactate and carbon dioxide is facilitated by this adaptation. Thus, there is no doubt about the structural importance of angiogenesis for enhancing the function of exercising skeletal muscle.

However, the importance of angiogenesis is reduced in magnitudes when the blood supply of the skeletal muscle is limited by occlusion of a major artery. Here arteriogenesis is the dominant factor. Preexisting collateral arterioles grow by mitotic cell division and after DNA replication of endothelial and smooth muscle cells. The developing arteries are capable of replacing a substantial amount of blood flow and therefore provide the prerequisite for survival of the distal tissues.

Early studies in cats and in rats demonstrated the increase in blood flow through these collateral arteries in response to endurance exercise. The increase in shear stress and the release of growth factors due to endurance exercise probably mediate this response. Early studies in dogs suggested a beneficial effect of exercise on growth of coronary collateral arteries. However, this could not be confirmed by investigation of collateral dependent blood flow utilizing a rigidly standardized isolated heart model. Exercise leads to cardiovascular adaptations that most likely caused these false positive results. Nevertheless, the improvements in cardiac function are impressive and of importance for patients suffering from myocardial infarction. Data obtained in pigs do not conclusively prove the enhanced development of collateral coronary arteries due to exercise. However, exercise was shown to induce arterial growth in normal myocardium of pigs. There are only a few studies in dogs and pigs investigating the role of exercise in PAOD.

More recent studies in rats demonstrated the potential of growth factor application in addition to exercise. When comparing to single exercise or growth factor treatment the combination of both is much more effective when considering the increase in hindlimb blood flow and muscle performance.

Our latest study in a transgenic mouse model of PAOD indicates a significant treatment interaction between FGF–2 and regular physical activity (see also chapters 5.3. and 6.3.). We observed a different collateral growth pattern with formation of a dense vascular network. Although to a lesser extent, the same pattern was found in trained nontransgenic mice suggesting that physical activity induced branching and growth of the preexisting collateral arterioles.

This response appears to be more effective than just the enlargement of a few collateral arteries exhibiting the corkscrew growth pattern.

In conclusion, there is enormous evidence about the induction of angiogenesis by regular physical activity. The expression of various growth factors is mediating this process. Enhanced arteriogenesis due to physical activity has been demonstrated in some animal models but not in others. Although it is evident that patients with cardiovascular diseases benefit from endurance exercise, enhanced arteriogenesis has not yet proven to be one of the main reasons for this beneficial effect. Recent studies in rats and mice have demonstrated that growth factor application causes a significant treatment interaction with endurance exercise. It is of immense importance to extent these first encouraging studies to other animal models and finally to the human patient. This powerful approach could potentially cure thousands of patients suffering from cardiovascular diseases.

4. Methods

4.1. Animals

The present studies were performed with permission of the State of Hessen, Regierungspräsidium Darmstadt, according to section 8 of the German "Law for the protection of animals." The investigations conformed with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892]. All mice were housed in standard cages, maintained on a 12 hour light and 12 hour dark cycle, and received water and chow ad libitum.

4.1.1. FGF–1 Overexpressing Mice

For experiments of simulated ischemia 18 mice with cardiac-specific overexpression of FGF–1 (FGF–1 transgenic) were used.

A 2.2 kb *Eco*RI fragment of clone pHBGF1.3A, including the coding and the 3' untranslated sequence of human FGF–1 but lacking any signal sequence, was ligated between the SV40 large intron and the 2.2 kb mouse MLC2v promoter. A CMV enhancer was positioned upstream from the MLC2v promoter, which provided the cardiac-specific expression (plasmid CMV/MLC2v/FGF) (Figure 3). The nontransgenic littermates (n=18) served as control mice. The mice were generated at the Max-Planck-Institute, Department of Experimental Cardiology.

The same strain of FGF–1 transgenic mice was used for determination of a pressure-flow-relationship (eight FGF–1 transgenic mice and seven nontransgenic control mice). The mice were 12 weeks old at the onset of the studies. (For detailed description of the generation of FGF–1 transgenic mice see [48].)

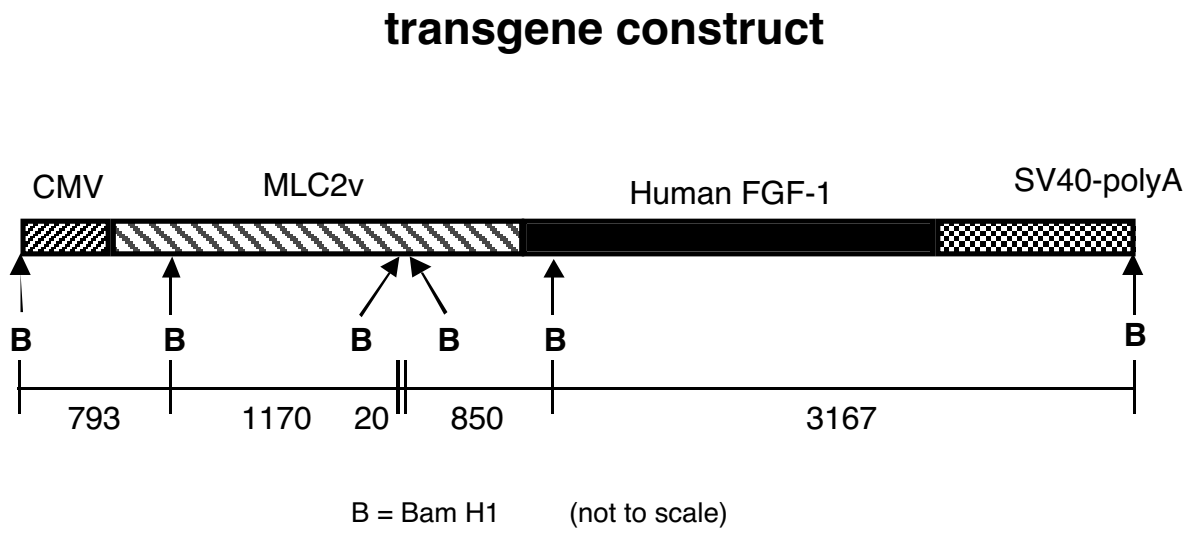


Figure 3. *Schematic drawing of the construct used for generation of FGF–1 transgenic mice. The human full-length FGF–1 cDNA was linked to the murine MLC2v promoter for heart-specific expression and to the SV40 large intron and polyA+ sequence for correct processing. The CMV enhancer was linked upstream to the MLC2v promoter for enhanced transgene expression. BamHI restriction sites used for Southern blotting and the length in base pairs of the fragments are indicated (not to scale).*

4.1.2. FGF–2 Overexpressing Mice

To determine the effects of FGF–2 and regular physical activity on exercise capacity of mice with unilateral femoral artery occlusion 12 male mice with general overexpression of FGF–2 (FGF–2 transgenic) were used.

A constitutive phosphoglycerate kinase promoter and polyadenylation sequence were used for general overexpression of FGF–2. A human FGF–2 cDNA was ligated to the promoter with a short intronic sequence inserted between the promoter and the FGF–2 cDNA in plasmid Blueskript II SK. The founder generation of FGF–2 transgenic mice was kindly provided by Dr. J. Douglas Coffin of the University of Montana (for detailed description of the generation of FGF–2 transgenic mice see [228]). Breeding of the experimental animals was conducted at the Max-Planck-Institute, Department of Experimental Cardiology. The nontransgenic littermates (n=12) were used as control mice. At the onset of the study all mice were 12 weeks old.

4.1.3. MCP–1 Overexpressing Mice

To determine the changes in exercise capacity after the onset of chronic heart failure due to myocarditis 10 male mice with cardiac-specific overexpression of MCP–1 (MCP–1 transgenic) were used.

A 1.9 kb *Eco*RI fragment of mouse JE-MCP–1 gene cloned into pUC19 plasmid was ligated to the Sa/I–KpnI–digested Myhca clone. The α–cardiac myosin heavy chain gene (Myhca) promoter was responsible for the cardiac-specific expression (for detailed description of the generation of MCP–1 transgenic mice see [94]). The founder generation of MCP–1 transgenic mice was kindly provided by Dr. Pappachan E. Kolattukudy of the Ohio State University. Breeding of the experimental animals was conducted at the Max-Planck-Institute, Department of Experimental Cardiology. The nontransgenic littermates (n=5) were used as control mice.

At the onset of the study all mice were 16 weeks old. Every four weeks, one MCP–1 transgenic mouse with an exercise capacity representing the average exercise capacity of the transgenic mice was used for histological analysis of the heart.

4.1.4. Nontransgenic Mice

For determination of the change in exercise capacity immediately after bilateral femoral artery occlusion male Balb/C (n=12), sv129 (n=12), or C57Bl/6 (n=12) mice were used. The mice were obtained from Charles River Laboratories (Sulzfeld, Germany) at an age of 11 weeks and after an habitation period of one week immediately used for the studies. Six mice of each strain with unilateral femoral artery occlusion performed at the same day were used for determination of the recovery of collateral dependent blood flow by Laser Doppler Imaging.

4.2. Simulated Ischemia

4.2.1. Isolation of Mouse Ventricular Cardiac Myocytes

Ventricular cardiac myocytes of FGF–1 transgenic and control mice were isolated as previously described for rats [229] with a few adoptions.

In brief, the mice were anesthetized by intra peritoneal (i.p.) injection of a mixture of ketamine (10 %, Medistar) and xylazine (Serum-Werk Bernburg AG). Five IU of heparin (Liquemin N 25000, Roche) were administered.

Under a binocular microscope (Leica) the thorax was opened, the heart quickly excised and placed in ice-cold perfusion buffer (110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM HEPES, 11 mM glucose, pH 7.4). The ascending aorta was cannulated with a polyethylene catheter (inner and outer diameter 0.40 and 0.80 mm, respectively) (Portex), which was connected with a 26 gauge hypodermic-needle (Sterican) to a 1 ml single-use syringe (Braun) filled with perfusion buffer and 5 IU heparin. The tip of the polyethylene catheter was placed approximately 2 mm distal to the aortic valve. The catheter was fixed with a 5-0 surgical suture. Care had to be taken that the catheter, needle, and syringe were free of air bubbles.

Next, the heart was retrogradely perfused with the content of the syringe. The perfusion lasted approximately 1 min and was used to control the placement of the catheter tip, to confirm the absence of any leakage, to remove blood from the coronary system, and to look for damage of the heart and air bubbles in the coronary system.

Thereafter, the heart was connected to a Langendorff-perfusion-apparatus (Figure 4) and retrogradely perfused with perfusion buffer gassed with 95% O_2 and 5% CO_2. Four minutes later perfusion was switched to 0.03% collagenase (CLS 2, Worthington Biochemical Corp), 0.004% pronase (Boehringer), 0.005% trypsin (Sigma), and 0.04 mM $CaCl_2$ for 25 min.

Then the heart was disconnected from the Langendorff-perfusion-apparatus, minced in the collagenase solution containing 1.2% BSA (Sigma), gently shaken at 37°C for 5 min, filtered through a nylon mesh and centrifuged at 15g for 5 min. The pellet was then carefully resuspended in perfusion buffer containing 0.1 mM $CaCl_2$ and centrifuged at 15g for 3 min. This step was repeated twice with perfusion buffer first containing 0.5 mM $CaCl_2$ and then containing 1.0 mM $CaCl_2$ and 4% BSA. The cells were resuspended in medium 199 (Sigma)

containing 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 0.1 mM insulin, 10 mM cytosine arabinoside, and 100 IU penicillin–streptomycin.

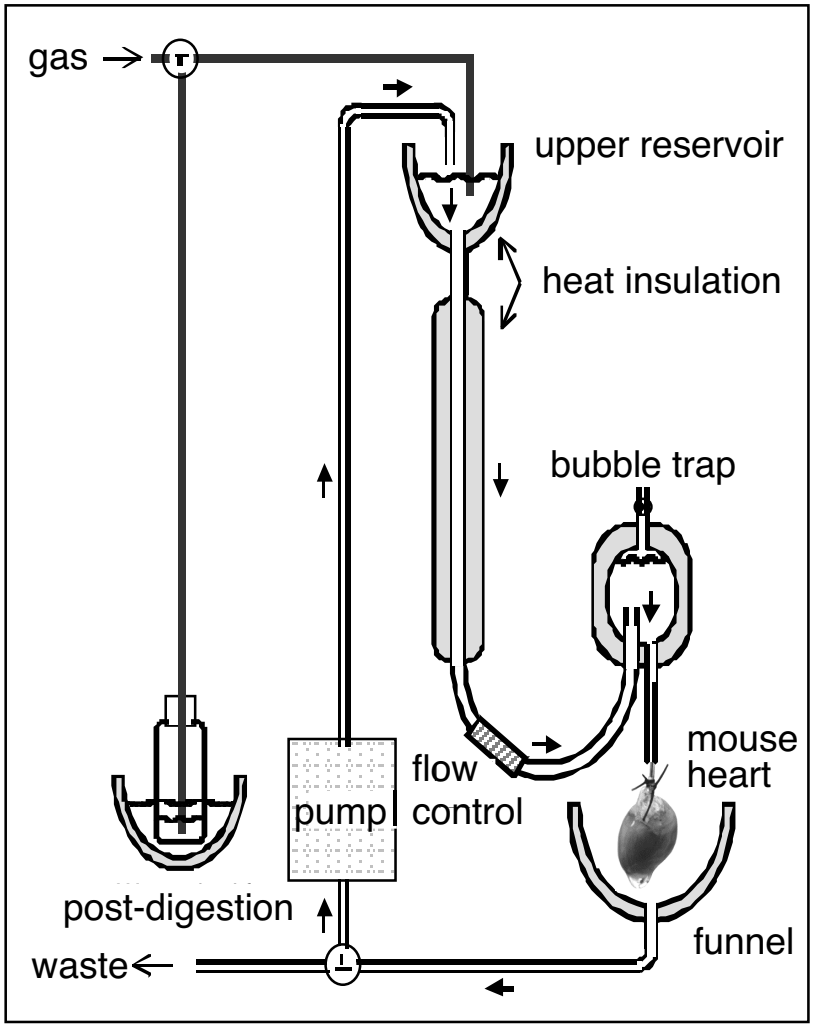


Figure 4. *Schematic drawing of the Langendorff-perfusion-apparatus used for isolation of ventricular cardiac myocytes from the mouse heart. The initial volume of perfusion buffer in the upper reservoir was 100 ml. Total retrograde perfusion lasted for approximately 30 min, making a recirculation of the perfusion buffer unnecessary.*

Cardiac myocytes were plated in 35 mm culture dishes (Falcon), coated with 5 μg/ml laminin (Sigma) and 10 μg/ml fibronectin (PromoCell). No FCS was added to the culture medium. Ventricular cardiac myocytes were allowed to attach to the dish for 3h before experiments of simulated ischemia were started (Figure 5).

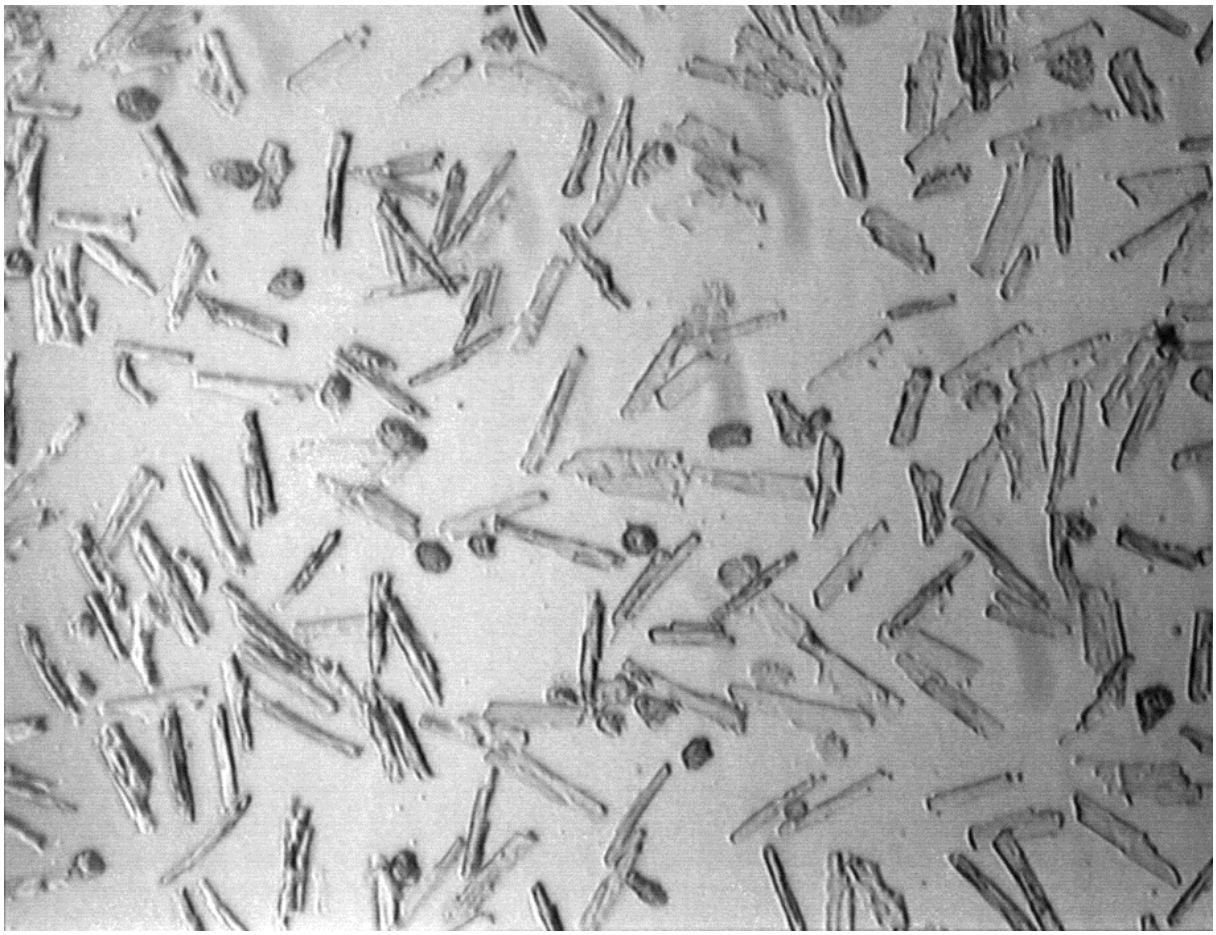


Figure 5. *Isolated ventricular cardiac myocytes of an adult FGF–1 transgenic mouse. Representative picture of attached cardiac myocytes plated in the appropriate density.*

4.2.2. Simulated Ischemia

Ischemia of adult mouse ventricular cardiac myocytes was simulated by modifying experimental protocols described for different species [230-233].

First, a glucose-free modified tyrode solution (110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4) was gassed with 95% N_2 and 5% CO_2 for 30 min. The anoxic solution was kept at 37°C in a tightly closed syringe (1 ml). Then the dishes were carefully washed three times with a glucose-free modified tyrode solution (110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4). After completion of the last wash the anoxic glucose-free tyrode solution was quickly released into the dish, which was immediately placed in gas tight chambers of own design (Figure 6). The chambers were closed fast and the

cardiac myocytes were incubated at 37°C in an environment of 95% N_2 and 5% CO_2 for 4, 8, and 12 hours.

Reoxygenation was achieved by switching to medium 199 and incubation with 21% O_2, 74% N_2, and 5% CO_2 at 37°C for 4 hours. Time matched control myocytes were incubated in an environment of 21% O_2, 74% N_2, and 5% CO_2 at 37°C.

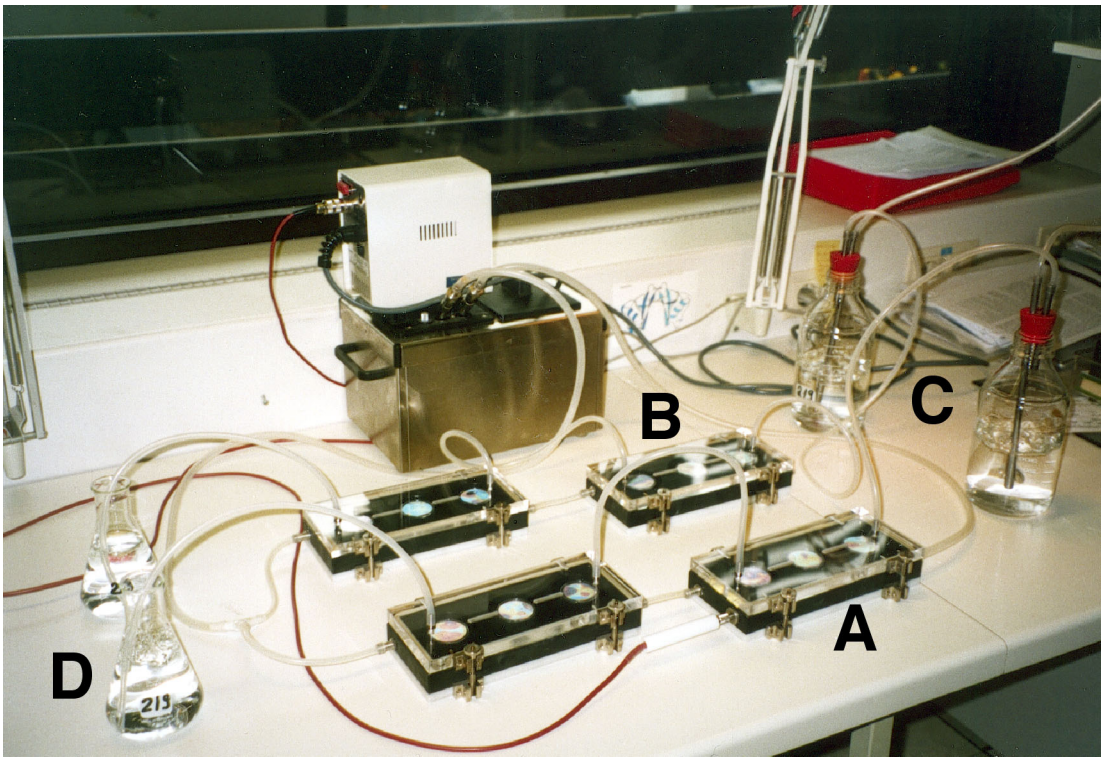


Figure 6. *Setup for experiments of simulated ischemia. Anoxia chambers (A) are perfused with 95% N_2 and 5% CO_2. Normoxia chambers (B) are perfused with 21% O_2, 74% N_2, and 5% CO_2. Note that the bottom of all chambers is filled with water to ensure sufficient humidity. Both gas mixtures are humidified separately (C). The gas outlets are placed in a beaker (D) filled with water column of 10 cm to visually control the flow rate and to prevent a back stream of room air.*

The activities of lactate dehydrogenase (LDH) and creatine kinase (CK) in the supernatant were determined after simulated ischemia, reoxygenation and lysis of the cells with 1% TritonX-100 (Sigma). Therefore, at each timepoint the supernatant was carefully taken from each dish, collected in a 1.5 ml microcentrifuge tube (500PP, Greiner), and centrifuged 2000 rpm for 5 min. Then two 300 µl samples of supernatant of each tube were carefully taken,

collected in 1.5 ml microcentrifuge tubes, immediately frozen in liquid nitrogen, and stored at –80°C until further analysis. The remaining volume of the initial supernatant contained a few broken cardiac myocytes and cellular debris and was discarded in order not to disturb the measurements. For lysis of the cells medium 199 with Triton-X (1% final concentration) was applied to the cells. The dishes were then placed on a shaker (Schüttler MTS4, IKA) and gently moved at 200 rpm for 30 min.

The release of enzymes was calculated as the percentage of activity after ischemia / reoxygenation versus total activity (simulated ischemia + reoxygenation + TritonX-100 extracts). The activities of LDH and CK were determined by a Hitachi 917 Automatic Analyzer (Hitachi) according to methods of the German Society for Clinical Chemistry 1970 [234] and 1993 [235], respectively. The hearts of six FGF–1 transgenic mice and six nontransgenic control mice were used per timepoint of simulated ischemia.

4.3. Determination of a Flow-Pressure-Relationship

The hearts of eight FGF–1 transgenic mice and of seven nontransgenic littermates used as control mice were quickly dissected, cannulated through the ascending aorta and retrogradely perfused as described above (chapter 2.1).

Thereafter, the catheter was connected to a perfusion apparatus of own design, which allowed a pressure and temperature controlled retrograde perfusion of the mouse heart. Perfusion buffer was supplemented with 0.01% adenosine to achieve maximal vasodilation and kept at a temperature of 37°C. Four different perfusion pressures were applied: 80 mmHg, followed by 100, 122, and 142 mmHg for 10 minutes each.

The coronary perfusates were collected in 50 ml tubes (Falcon). After centrifugation at 2000 rpm the volume of the coronary perfusate was determined. Finally, the hearts were weighed on a scientific scale (BP 211 D, Sartorius). The coronary flow was expressed in terms of milliliters of coronary perfusate per 150 mg of heart weight.

Preliminary experiments showed that addition of BSA or any other protein to the perfusion buffer was not necessary. The coronary flow at 80 mmHg was the same before and after the regular 40 min perfusion period indicating that no vascular leakage, possibly due to a lack of protein in the perfusion buffer, occurred.

4.4. Femoral Artery Occlusion and Blood Flow Measurements

Mice were anesthetized by injection (i.p.) of a mixture of ketamine (10 %, Medistar) and xylazine (Serum-Werk Bernburg AG). The right femoral artery was ligated proximal to the orifices of the arteria poplitea but distal to the branching-point of the arteria profunda femoris (Figure 7). Blood flow measurements were performed before, immediately after, three, seven, 21, and 28 days after occlusion, using the same anesthesia protocol. At the end of the study the mice were euthanized and the descending thoracic aortas were cannulated for subsequent perfusion fixation of the hindlimbs.

A red Laser Doppler Imager (MLDI 5063, Moor Instruments Ltd.) was used to detect blood cell motion in the foot to estimate relative blood flow. Relative score of vessels were compared after the background subtracting.

Preliminary experiments showed that blood flow results obtained by LDI are dependent on the ambient temperature. Therefore, in order to standardize measurements, anesthetized mice were kept for 5 min in a climatized chamber of own design at 37°C before the measurements were performed. Special care was taken that the measured paws were clean and dry.

Blood flow measurements by LDI were performed in sedentary FGF–2 transgenic and nontransgenic control mice on following time points: before right femoral artery occlusion, immediately thereafter and on days 3, 7, 14, 21, 28, and 39 after occlusion. Trained mice were only measured before, immediately after, and 39 days after the initial surgery to avoid disturbance of the exercise protocol due to the anesthesia. The last training session was completed at day 36 to ensure a full recovery of two days before the final measurements. LDI measurements of the whole feet were made at a pixel resolution of 256x256 and at a scan rate of 4 ms/pixel for a 1.7 by 3 cm area. Dead mice were used to determine the background level separately for each strain for subsequent subtraction. The right-to-left, i.e. occluded-to-nonoccluded side, perfusion ratios were calculated for each timepoint and mouse (Figure 8).

To determine gastrocnemius blood flow by MRI a flow phantom consisting of four tubes connected in series with inner diameters of 0.40 mm, 0.58 mm, and two of 1.46 mm was evaluated in an eight-leg 2.8 cm diameter by 3.2 cm length lowpass birdcage coil. Sufficiently long tubing between the flow tubes allowed for equilibration of exited spins before entering the detection and excitation plane.

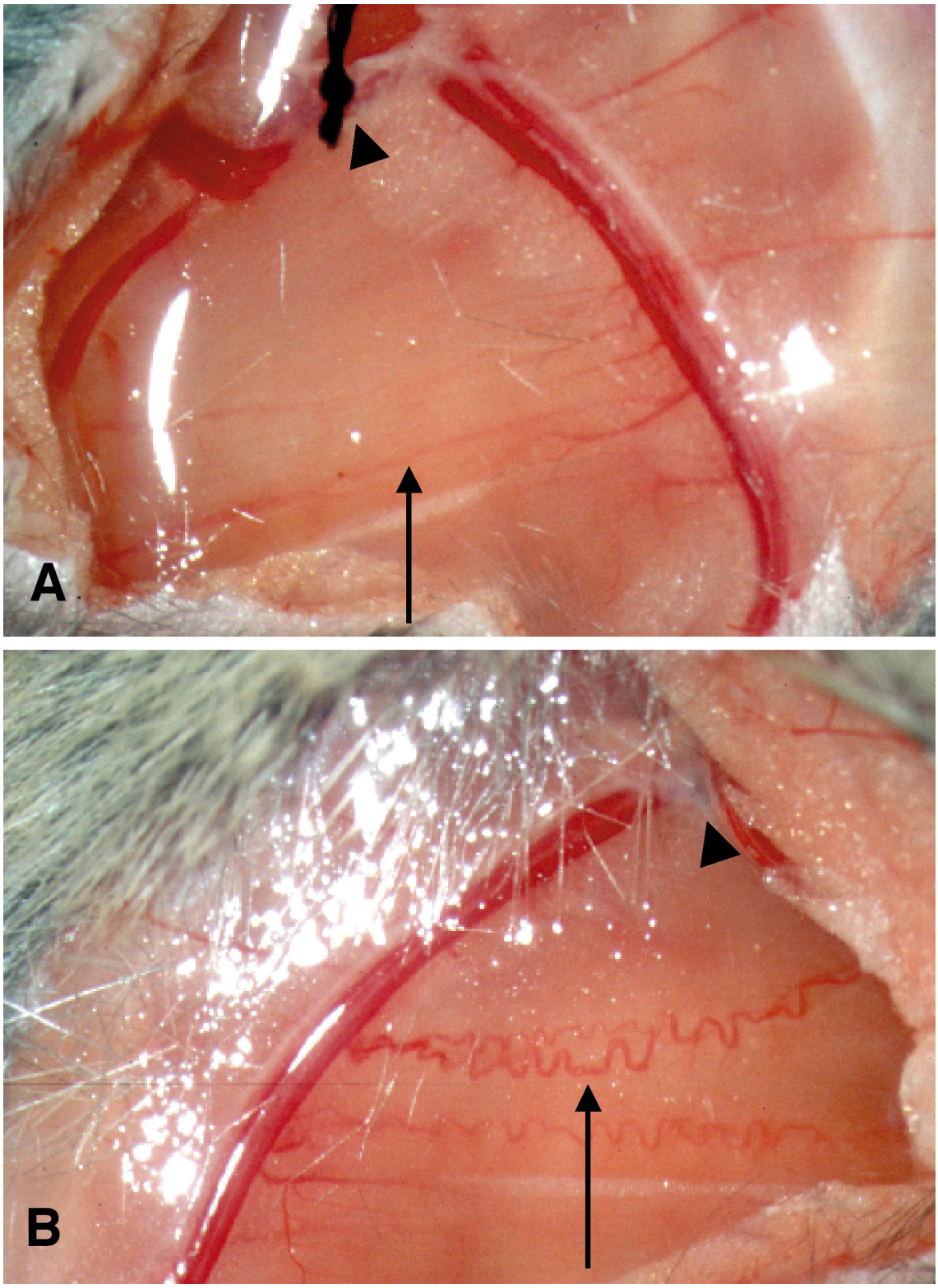


Figure 7. *A) Acute occlusion (arrowhead) of the left femoral artery. Note the preexisting superficial collateral arterioles (arrow). B) Growing collateral arteries (arrow) seven days after occlusion of the right femoral artery (arrowhead). The collateral arteries grow in both diameter and length resulting in the typical corkscrew growth pattern.*

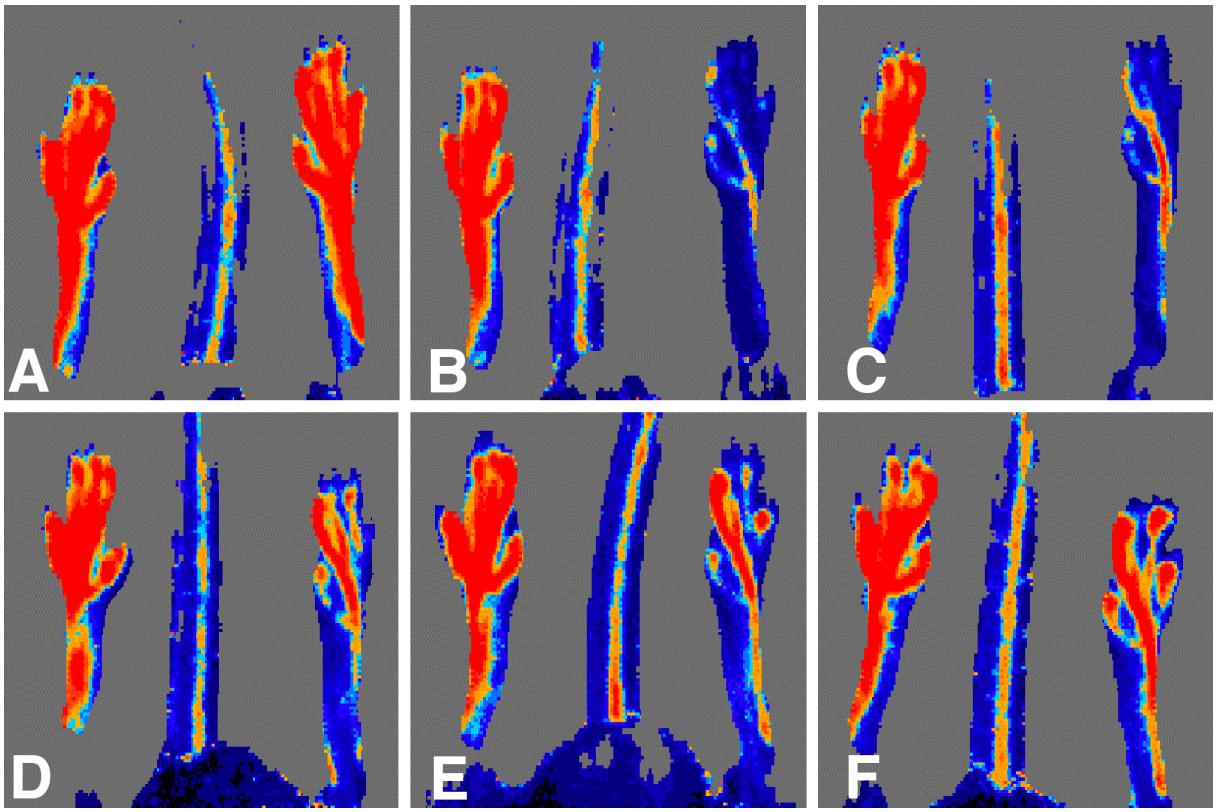


Figure 8. *Representative time course of foot blood flow recovery after right femoral artery occlusion of a Balb/C mouse determined by Laser Doppler Imaging (LDI). A) immediately before occlusion, B) immediately after occlusion, C) three days, D) seven days, E) 14 days, and F) 21 days after occlusion of the right femoral artery.*

Time-of-flight images were acquired in a Bruker PharmaScan 70/16 7.0 Tesla imager for a square field of view of 1.28 cm for a two-dimensional matrix of 128x128. Slice planes of 0.62 mm, 1 mm, and 1.5 mm were acquired perpendicular to the axially flow of aqueous 1.0 mmol/l copper sulfate solution used to simulate T_1 and T_2 for blood. Flow rates were changed by altering the height of a supply water reservoir and restricting the water outflow. Linear flow through the three tubes of different diameters was calculated by measuring the mass of solution for the determined time period. The acquisition of the image plane was done by acquiring single lines in k-space every 23 ms with 90° pulses and echo times of 8 ms. In a steady state with no flow, a loss of magnetization is observed due to the fast repetition time and large pulse angle. However, under conditions of inflowing fluid the magnetization is determined by the amount of fluid that enters the slice between detection pulses. The theoretical maximum occurs when the linear flow equals or exceeds the length of the detection slice per unit of time between detection slices. For laminar flow this does not hold true since the fluid in the center of the tube moves at a much faster rate than near the

walls. This can be observed in images with slow flow rates. The intensity in the center volume is higher than the edges. In nonlinear tortuous vessels streaming has also been characterized and observed. The smaller tubes of the flow phantom showed similar effects. Streaming occurred preferential at the edges of the tubes. Increasing slice thickness results in phase artifacts from greater field inhomogenities, manifested as concentric rings in the cross-section. These artifacts, however, did not significantly affect the results at the slow flow rates. Gastrocnemius blood flow was determined at day 39 after femoral artery occlusion.

At the end of the study all mice were euthanized. The thorax was opened and the descending aorta was cannulated as described for the ascending aorta (see chapter 4.1). Then the catheter was connected to a perfusion apparatus of own design, which allowed a pressure and temperature controlled perfusion of the distal vessels. The perfusion buffer was supplemented with 0.01% adenosine to achieve vasodilation. The distal vessels were perfused with perfusion buffer at a temperature of 37°C and a pressure of 100 mmHg until they were free of blood. Afterwards, both hindlimbs were perfusion-fixed with 2% paraformaldehyde in perfusion buffer for 10 minutes at a pressure of 100 mmHg.

Following perfusion fixation a contrast medium (bismuth chloride in 5% gelatin) was infused and the hindlimbs were immersed in ice. Angiographies were acquired using a Machlett-Balteau X-ray source (set at 20 kV /8 mA). Superficial collateral arteries visible under the operating microscope were photographed and sampled for ultrastructural studies and histological analysis. Furthermore, skeletal muscle samples containing the deep collateral arteries and the gastrocnemius muscles were sampled for histological analysis. The postmortem angiographies were individually evaluated by two independent observers unaware of the sample identities.

4.5. Training of Mice with Unilateral Femoral Artery Occlusion

Two days after unilateral occlusion of the femoral artery 12 FGF–2 transgenic animals and their 12 nontransgenic littermates that were used as control mice were randomly separated into six sedentary mice and six mice that were trained for five weeks. The mice were trained using a progressive, moderate intensity endurance running regime on the mouse treadmill as follows:

Day 0: Occlusion of the right femoral artery.

Day 1: Rest.

Day 2: Rest.

Day 3: Running at an inclination of 9° and a speed of 10 m/min for 30 min.

Day 4: Running at an inclination of 9° and a speed of 11 m/min for 35 min.

Day 5: Running at an inclination of 9° and a speed of 12 m/min for 40 min.

Day 6: Rest.

Day 7: Running at an inclination of 9° and a speed of 13 m/min for 45 min.

Day 8: Running at an inclination of 9° and a speed of 14 m/min for 50 min.

Day 9: Running at an inclination of 9° and a speed of 15 m/min for 55 min.

Day 10: Running at an inclination of 9° and a speed of 16 m/min for 55 min.

Day 11: Running at an inclination of 9° and a speed of 17 m/min for 60 min.

Day 12: Running at an inclination of 9° and a speed of 18 m/min for 60 min.

Day 13: Rest.

Day 14: Running at an inclination of 9° and a speed of 19 m/min for 60 min.

Day 15: Running at an inclination of 9° and a speed of 20 m/min for 60 min.

Day 16: Running at an inclination of 9° and a speed of 21 m/min for 60 min.

Day 17: Running at an inclination of 9° and a speed of 22 m/min for 60 min.

Day 18: Running at an inclination of 9° and a speed of 23 m/min for 60 min.

Day 19: Running at an inclination of 9° and a speed of 24 m/min for 60 min.

Day 20: Rest.

Day 21: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 22: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 23: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 25: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 26: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 27: Running at an inclination of 9° and a speed of 30 m/min for 60 min.

Day 28: Rest.

Day 29: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 30: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 31: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 32: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 33: Running at an inclination of 9° and a speed of 25 m/min for 60 min.

Day 34: Running at an inclination of 9° and a speed of 30 m/min for 60 min.

Day 35: Rest.

Day 36: Determination of exercise capacity.

Day 37: Rest.

Day 38: Rest.

Day 39: Final measurements and tissue harvesting.

The first exercise session was initiated at day 3 post ligation. The inclination was set to 9° and a speed of 10 m/min was chosen. The first exercise session lasted for 30 min. Thereafter, the speed and the duration were increased by 1 m/min and 5 min, respectively, until the mice were able to run up a 9° inclination at a speed of 25 m/min for 60 min (Figure 9). This level was achieved at day 21 of the study. During the first week, the mice were submitted to three consecutive exercise sessions followed by one day of rest. From there on, mice were trained six times per week followed by one day of rest. After the mice were capable to run at the final workload, the day before the day of rest was used for a more strenuous exercise session at a speed of 30 m/min. If a mouse exhibited signs of fatigue the exercise session was terminated. The final exercise capacity was determined at day 36 of the study after one day of rest. Thereafter, the animals were allowed to recover for two days before the final measurements of blood flow by LDI and MRI, angiography, and tissue harvesting.

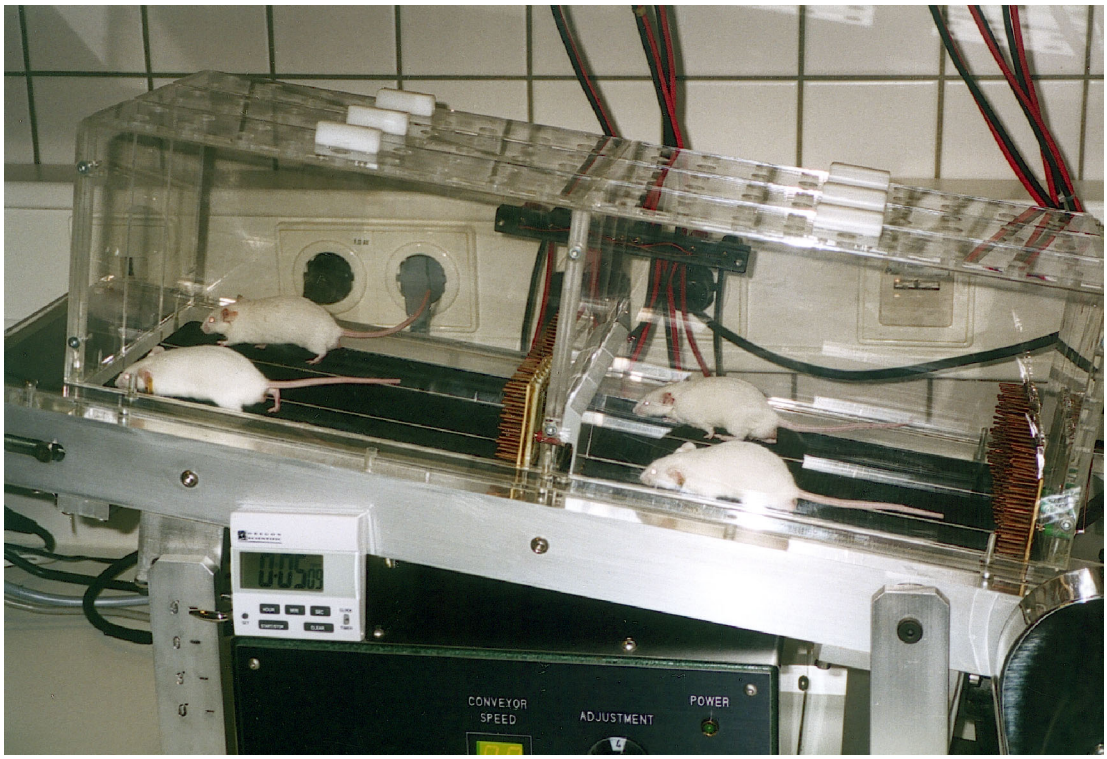


Figure 9. *Typical exercise session of FGF–2 transgenic mice and nontransgenic control mice with unilateral femoral occlusion. The mice are running at an inclination of 9° and a speed of 25 m / min for 60 min.*

4.6. Determination of Exercise Capacity

4.6.1. Time Course after Bilateral Femoral Artery Occlusion

Prior experiments revealed that mice with bilateral femoral artery occlusion are not able to perform the standard exercise test until seven days after the initial operation. Therefore, determination of the time course of exercise capacity changes after bilateral femoral artery occlusion was started seven days after the initial operation and was repeated every seven days until day 28.

All mice were initially familiarized with the treadmill. During the week before the operation they ran up an inclination of 3° at a speed of 10 m/min for 10 min. This was repeated twice after a break of one day.

The exercise protocol consisted of a graded running test on a mouse treadmill of own design (Figure 10), partially adopted from a rabbit treadmill of the physics workshop of the University of Melbourne in Australia. Each mouse initially ran up a 6° inclination at a speed of 20 m/min for 15 minutes (Figure 11). Thereafter,

the treadmill speed was increased by 5 m/min every 15 minutes until the animal reached the point of fatigue. The criterion used to determine fatigue was the inability of the mouse to keep pace with the treadmill. If the animal had lost its righting reflex, the end-point of fatigue was confirmed. The time from beginning of the exercise to the removal of the mouse from the treadmill was recorded.

The exercise tests were repeated every seven days until day 28 post-ligation. The same protocol was used for non-ligated control animals. At the end of the exercise test the performed work for each animal was calculated and expressed in Joule (J) as follows:

work (J) = body mass of animal (kg) × run distance (m) × [1 + 9.81m/s^2 × sine of inclination (°)] (for calculation see also [236]).

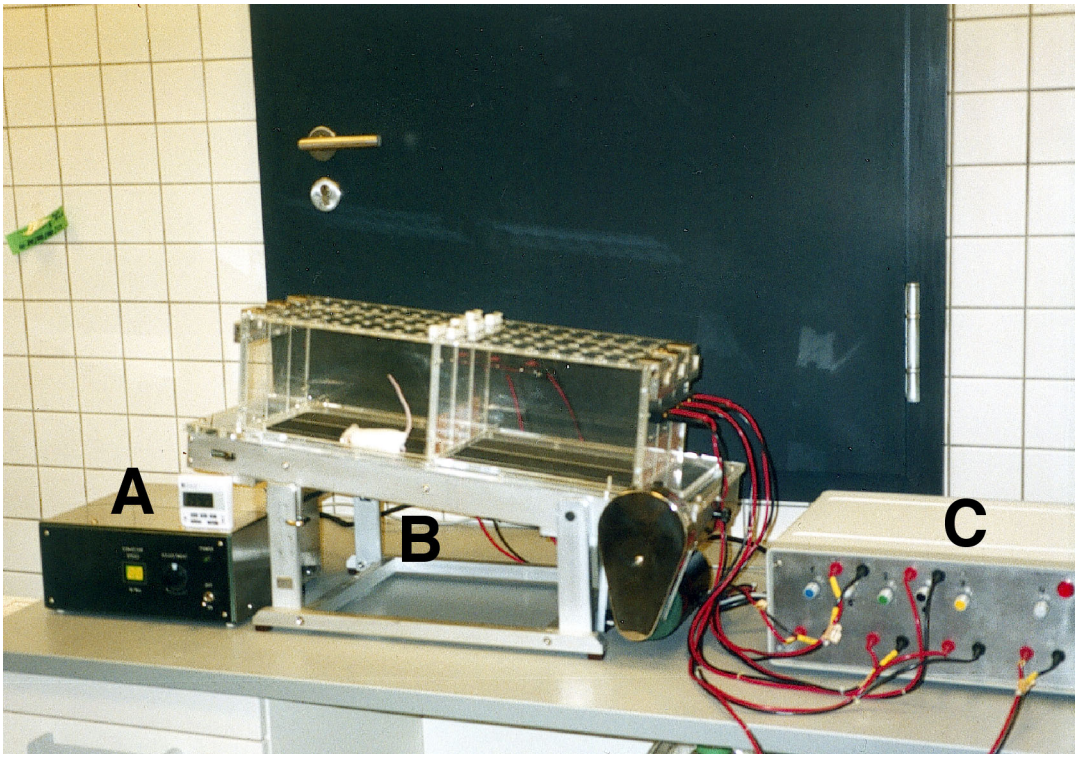


Figure 10. *Experimental setup for determination of exercise capacity. (A) treadmill-speed controller, (B) rodent treadmill, (C) electrical stimulation box*

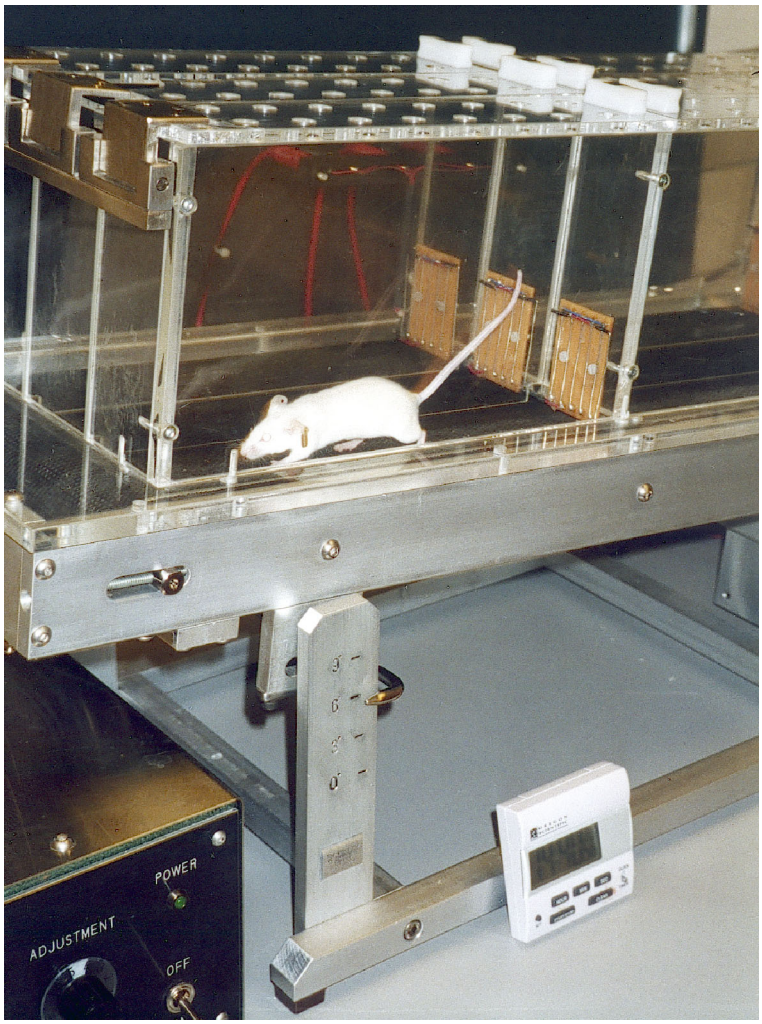


Figure 11. *Exercise test of a Balb/C mouse. The mouse is running in the initial stage of the exercise test protocol (inclination of 6° and a speed of 20 m/min) on the rodent treadmill.*

4.6.2. Time Course after the Onset of Congestive Heart Failure

The protocol of the exercise test used for MCP–1 transgenic mice was similar to the protocol described above (chapter 4.6.1.) with the exception that the first exercise test was performed at the age of 16 weeks. Thereafter, the tests were repeated every four weeks until all transgenic mice had died due to congestive heart failure. The same protocol was used for their nontransgenic littermates that served as control mice.

4.6.3. Trained Mice with Unilateral Femoral Artery Occlusion

FGF–2 transgenic animals and their nontransgenic littermates that used as control mice were randomly separated into sedentary mice and mice that were trained for five weeks. The exercise test was performed at day 36 after the initial operation. The protocol of the exercise test used for these mice was similar to the protocol described above (chapter 4.6.1.) with the exception that the inclination of the treadmill was 9°.

4.7. Statistical Analysis

After all data were analyzed for normal distribution and variance homogeneity a one–way analysis of variance (ANOVA) or a Student-Newman-Kuels post-hoc analysis revealed the significance of the groups differences. Differences at the $p<0.05$ level were considered significant.

5. Results

5.1. Simulated Ischemia

The release of LDH and CK from cardiac myocytes is caused by the loss of cell membrane integrity and is a sign of cell death. In clinical chemistry, both enzymes are used as markers of damage and death of various cell types including cardiac myocytes. The activity of these enzymes in the serum of patients provides valuable diagnostic tools for determination of the severity of myocardial infarction.

Creatine kinase release of FGF–1 transgenic myocytes was significantly decreased at four hours of simulated ischemia when compared to nontransgenic myocytes (10.86 ± 1.38% vs. 33.14 ± 2.26%, respectively, Figure 12, Table 1). At eight hours of simulated ischemia the release of CK of transgenic myocytes was still reduced when compared to nontransgenic myocytes (42.33 ± 1.99% vs. 60.48 ± 2.95%, respectively). However, at 12 hours of simulated ischemia there was no statistically significant difference between the CK release of transgenic and nontransgenic myocytes (76.65 ± 0.80% vs. 71.03 ± 1.53%, respectively). Under normoxic conditions there was no significant change in the release of CK of transgenic myocytes when compared with nontransgenic myocytes (2.54 ± 0.35% vs. 3.27 ± 0.37%, respectively) during the 16 hour experimental protocol.

Table 1. CK release of nontransgenic control myocytes and FGF–1 transgenic ventricular cardiac myocytes during 4h, 8h, and 12h of simulated ischemia and during the 16 hour experimental protocol under normoxic conditions.

Time point	Nontransgenic	FGF–1 Transgenic
Normoxia 16h	3.27 ± 0.37%	2.54 ± 0.35%
Ischemia 4h	33.14 ± 2.26%	10.86 ± 1.38%*
Ischemia 8h	60.48 ± 2.95%	42.33 ± 1.99%*
Ischemia 12h	71.03 ± 1.53%	76.65 ± 0.80%

Data are means ± SEM. * $p < 0.05$ vs. same time point of nontransgenic myocytes.

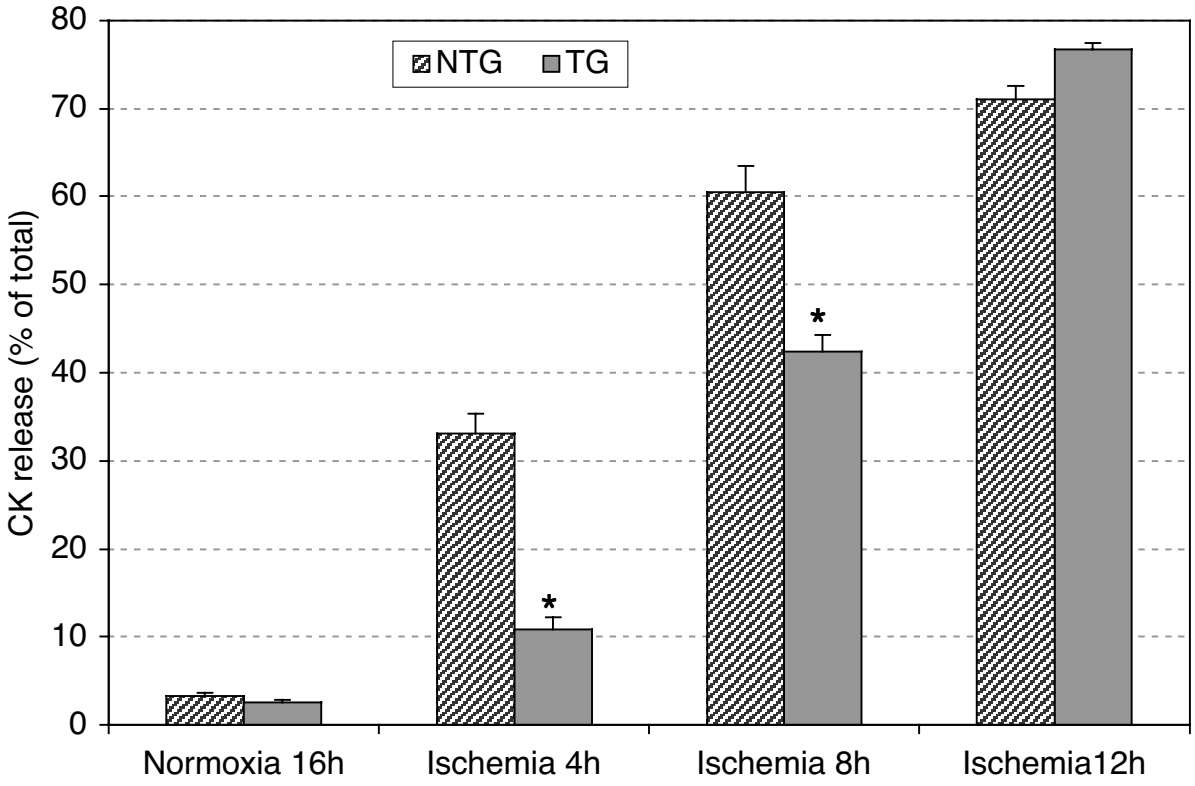


Figure 12. *CK release of nontransgenic control myocytes and FGF–1 transgenic ventricular cardiac myocytes during 4h, 8h, and 12h of simulated ischemia and during the 16h experimental protocol under normoxic conditions. Data are means ± SEM. * $p < 0.05$ vs. same time point of nontransgenic myocytes.*

Similar to the release of CK, LDH release of transgenic myocytes was decreased at four hours of simulated ischemia when compared to nontransgenic myocytes (33.59 ± 2.51% vs. 58.64 ± 3.02% respectively, Figure 13, Table 2). This difference was still apparent at eight hours of simulated ischemia (73.94 ± 3.18% vs. 86.87 ± 3.46%, respectively). However, at 12 hours of simulated ischemia the release LDH from transgenic myocytes was not significantly different when compared to LDH release from nontransgenic myocytes (90.98 ± 3.37% vs. 90.85 ± 1.56%, respectively).

Similar to the release of CK, the basal LDH release of transgenic and nontransgenic myocytes under normoxic conditions during the 16 hours of the experimental protocol was not significantly different (35.24 ± 1.86% vs. 34.69 ± 2.58%, respectively).

Table 2. LDH release of FGF–1 transgenic ventricular cardiac myocytes and nontransgenic control myocytes during 4h, 8h, and 12h of simulated ischemia and during the 16h experimental protocol under normoxic conditions.

Time point	Nontransgenic	FGF–1 Transgenic
Normoxia 16h	34.69 ± 2.58%	35.24 ± 1.86%
Ischemia 4h	58.64 ± 3.02%	33.59 ± 2.51%*
Ischemia 8h	86.87 ± 3.46%	73.94 ± 3.18%*
Ischemia 12h	90.85 ± 1.56%	90.98 ± 3.37%

Data are means ± SEM. * $p < 0.05$ vs. same time point of nontransgenic myocytes.

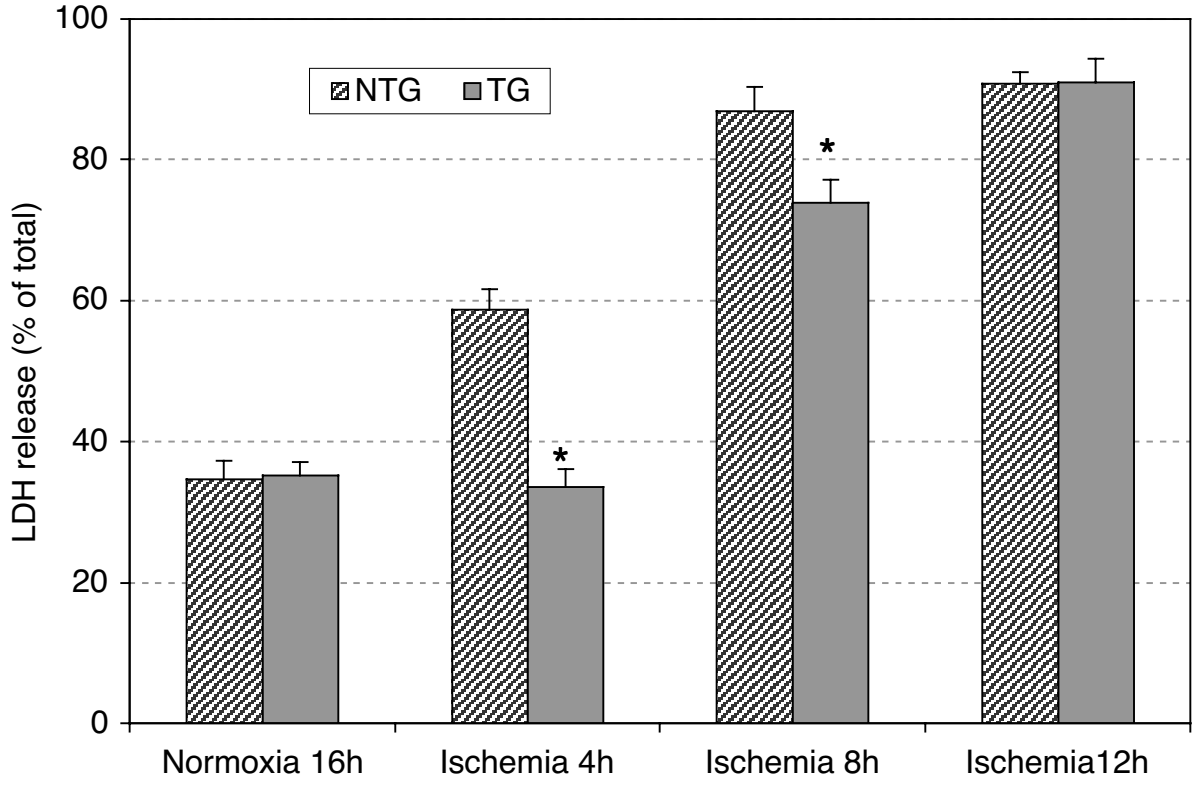


Figure 13. *LDH release of FGF–1 transgenic ventricular cardiac myocytes and nontransgenic control myocytes during 4h, 8h, and 12h of simulated ischemia and during the 16h experimental protocol under normoxic conditions. Data are means ± SEM. * $p < 0.05$ vs. same time point of nontransgenic myocytes.*

5.2. Determination of a Flow-Pressure-Relationship

There was no statistically significant difference in heart weight of FGF–1 transgenic mice when compared to nontransgenic control mice. The average heart weight was 150 mg. Therefore, the coronary flow was expressed in terms of milliliters of coronary perfusate per 150 mg of heart weight. The hearts of FGF–1 transgenic animals displayed a significantly increased coronary flow at all the pressures tested when compared to nontransgenic control mice. At a pressure of 80 mmHg coronary flow of transgenic hearts was 1.10 ± 0.04 ml/min/150mg (increased by 17%) while nontransgenic hearts had a coronary flow of 0.94 ± 0.05 ml/min/150mg (Figure 14, Table 3). At 100 mmHg coronary flow of transgenic hearts was increased by 19% when compared to nontransgenic hearts (1.46 ± 0.09 ml/min/150 mg vs. 1.23 ± 0.05 ml/min/150 mg, respectively). At 122 mmHg coronary flow of transgenic hearts was increased by 24% compared to nontransgenic hearts (1.81 ± 0.10 ml/min/150 mg vs. 1.46 ± 0.03 ml/min/150 mg, respectively). At 142 mmHg the increase in coronary flow of transgenic hearts was 33% when compared to nontransgenic hearts (2.14 ± 0.19 ml/min/150 mg vs. 1.61 ± 0.001 ml/min/150 mg, respectively).

The slope of the regression function corresponding to FGF–1 transgenic hearts was increased by 36% (b=0.0152) when compared to the slope of the regression function of nontransgenic hearts (b=0.0112) (Figure 15). The slope of the regression function for change in flow (Δflow) was significantly different from 0 (b=0.0040), demonstrating an elevated pressure-dependent increase in coronary flow of transgenic hearts.

Table 3. Coronary flow of nontransgenic controls and FGF–1 transgenic hearts at four different pressures (80, 100, 122, and 142 mmHg).

Coronary Flow (ml/min/150 mg) at	**Nontransgenic**	**FGF–1 Transgenic**
80 mmHg	0.94 ± 0.05	1.10 ± 0.04*
100 mmHg	1.23 ± 0.05	1.46 ± 0.09*
122 mmHg	1.46 ± 0.03	1.81 ± 0.10*
142 mmHg	1.61 ± 0.001	2.14 ± 0.19*

Data are means ± SEM. * $p < 0.05$ vs. nontransgenic hearts.

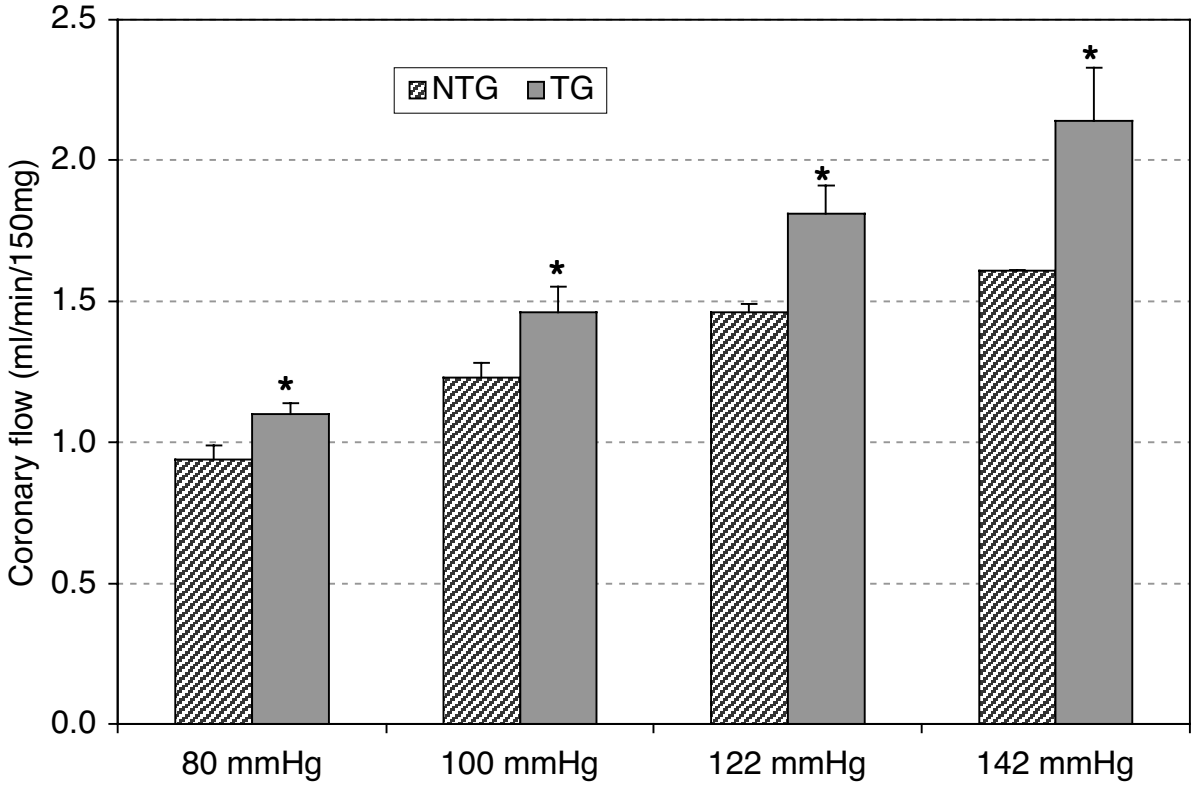


Figure 14. *Coronary flow of nontransgenic control hearts (NTG) and FGF–1 transgenic hearts (TG) at four different pressures (80, 100, 122, and 142 mmHg). Data are means ± SEM. * $p < 0.05$ vs. nontransgenic hearts.*

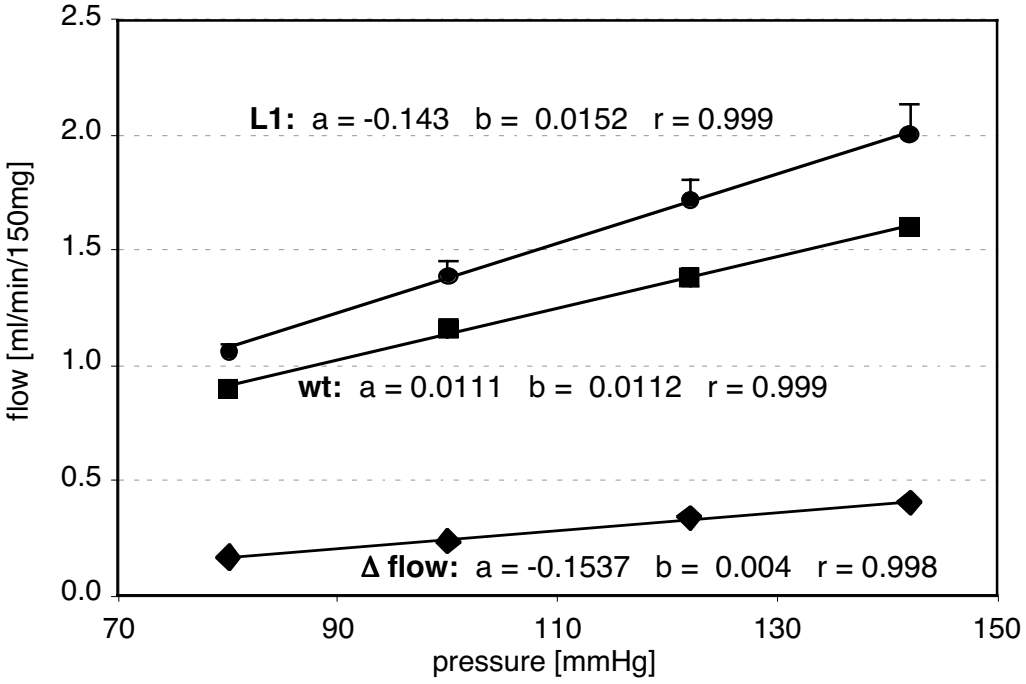


Figure 15. *Regression curves of coronary flow vs. pressure of nontransgenic control hearts (wt) and FGF–1 transgenic hearts (L1) at four different pressures (80, 100, 122, and 142 mmHg). (Δ flow = change in coronary flow between transgenic and nontransgenic hearts)*

5.3. FGF–2 Transgenic Mice with Unilateral Femoral Artery Occlusion

5.3.1. Progressive, Moderate Intensity Endurance Exercise Program

The intensity of the endurance exercise program was considered moderate because the mice did not exhibit signs of fatigue during the individual sessions. Signs of fatigue include frequent contacts with the grid (more than one contact per minute) and gait change to a gallop with exaggerated hops. Only during the two more strenuous exercise sessions (day 27 and day 34) some animals exhibited signs of fatigue by the end of the exercise session. These mice were removed from the treadmill and the program was completed in order to apply a strenuous stimulus to all mice.

A progressive character was chosen because it has been observed before that the exercise capacity of mice with bilateral femoral artery occlusion is dramatically reduced during the first week but recovers within 3 weeks to approximately 60%. The mice completed the first sessions without problems. In their cages the mice did not use their occluded leg for walking during the first week. However, while running on the treadmill the mice were forced to use the occluded leg properly. During the first week the mice were limping occasionally, especially at the end of the exercise session. During the second week of the exercise regime limping was observed less frequently and it disappeared during the third week. After the third week no difference in the leg motion between the occluded leg and the normal leg was observed.

It appeared that the occluded leg was darker and had a slightly blue color after the exercise sessions when compared to the normal leg. This effect was neither observed prior the exercise sessions in trained mice or in sedentary mice. Interestingly, the change in color persisted over the period of the experiment. The occluded leg looked different from the normal leg after each exercise session.

5.3.2. Collateral Dependent Blood Flow

Collateral dependent blood flow to the foot was determined by LDI. At day 39 post occlusion trained FGF–2 transgenic mice displayed significantly improved

foot blood flow when compared to sedentary nontransgenic and trained nontransgenic mice (0.97 ± 0.03 vs. 0.66 ± 0.10, 0.78 ± 0.06, respectively, Figure 16, Table 4). Sedentary FGF–2 transgenic mice showed a trend towards improved foot blood flow compared to sedentary nontransgenic mice (0.88 ± 0.06 vs. 0.66 ± 0.10, respectively). However, this trend did not achieve the level of significance.

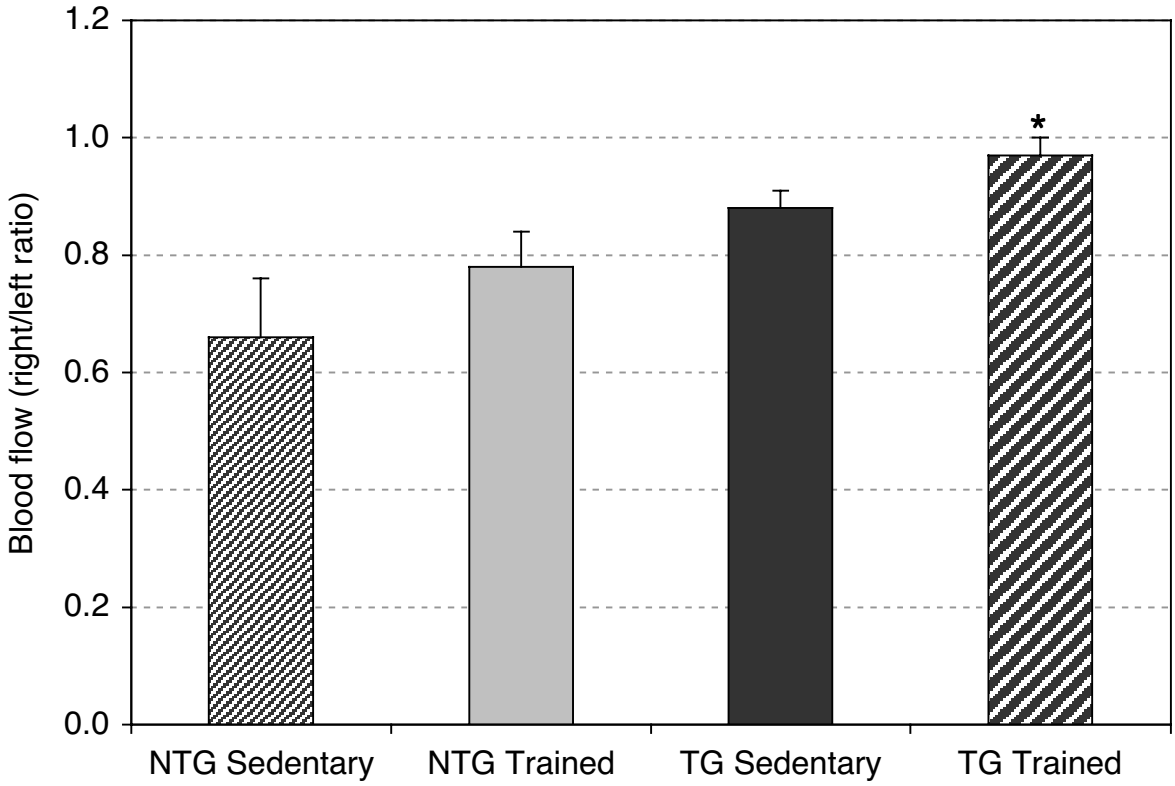


Figure 16. *Collateral dependent blood flow to the foot (determined by LDI) of sedentary nontransgenic (NTG Sedentary), trained nontransgenic (NTG Trained), sedentary FGF–2 transgenic (TG Sedentary), and trained FGF–2 transgenic (TG Trained) mice at day 39 post occlusion. Data are means ± SEM. * $p < 0.05$ vs. sedentary nontransgenic mice.*

Gastrocnemius blood flow of the occluded leg determined by MRI (Figure 18) revealed a significant increase in trained and sedentary transgenic mice when compared to sedentary nontransgenic mice (0.85 ± 0.06, 0.85 ± 0.10 vs. 0.60 ± 0.06, respectively, Figure 17, Table 4). Gastrocnemius blood flow of trained nontransgenic mice was similar to sedentary nontransgenic mice (0.61 ± 0.10 vs. 0.60 ± 0.06, respectively). However, changes in gastrocnemius blood flow of the normal leg were different from the changes observed in the occluded leg. There was a stepwise increase in blood flow from sedentary nontransgenic to trained nontransgenic to sedentary FGF–2 transgenic to

trained FGF–2 transgenic mice (0.83 ± 0.06, 0.93 ± 0.07, 1.00 ± 0.15, 1.16 ± 0.11, respectively). However, the only mice that demonstrated significantly increased gastrocnemius blood flow of the normal leg were the trained FGF–2 transgenic mice.

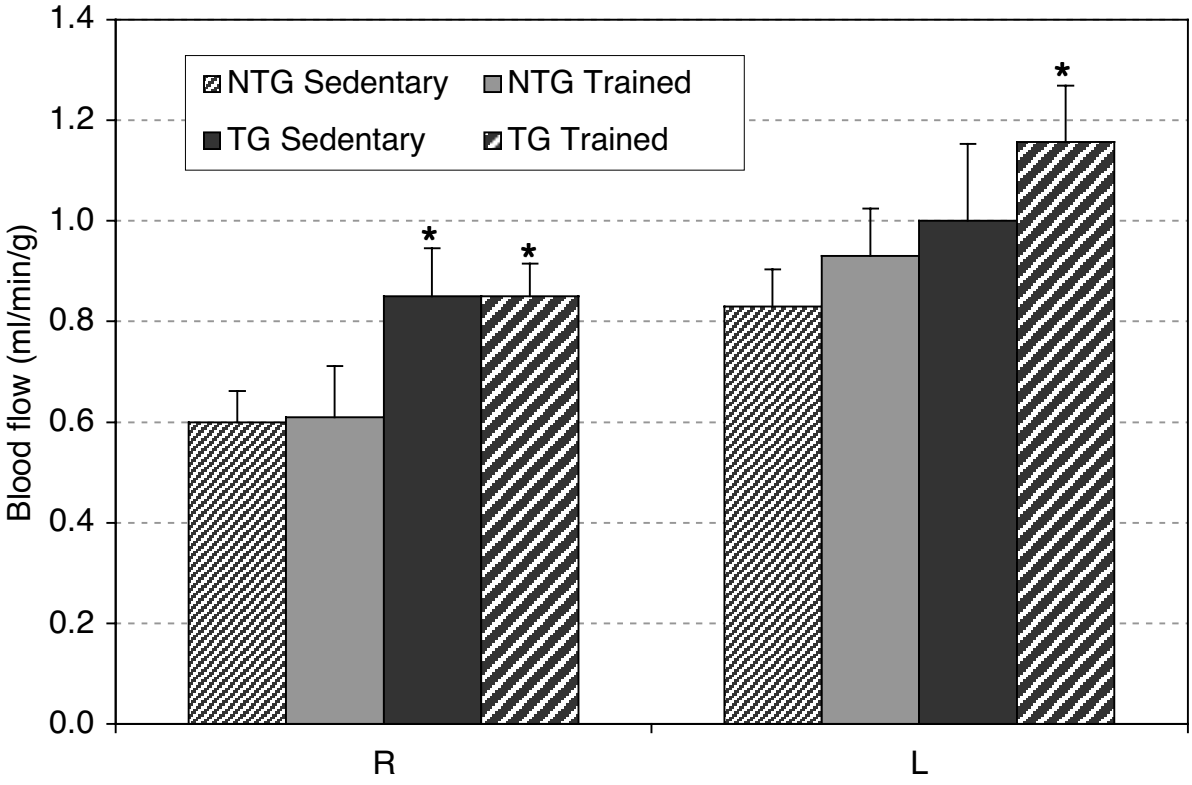
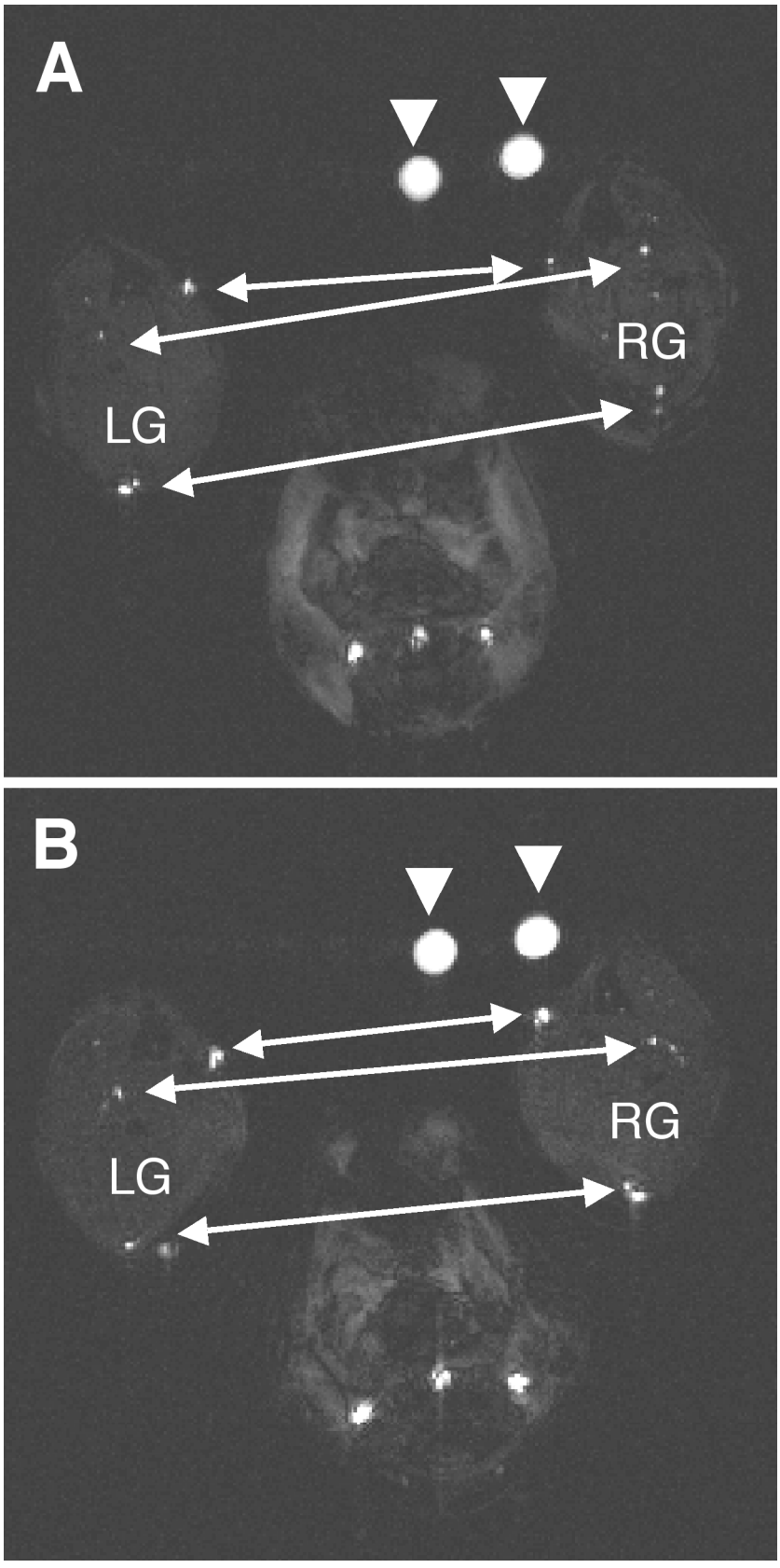


Figure 17. *Collateral dependent blood flow to the gastrocnemius (determined by MRI) of sedentary nontransgenic, trained nontransgenic, sedentary FGF–2 transgenic, and trained FGF–2 transgenic mice at day 39 post occlusion (R=right occluded leg, L=left normal leg). Data are means ± SEM. * $p < 0.05$ vs. sedentary nontransgenic mice.*


A
RG
LG
B
RG
LG

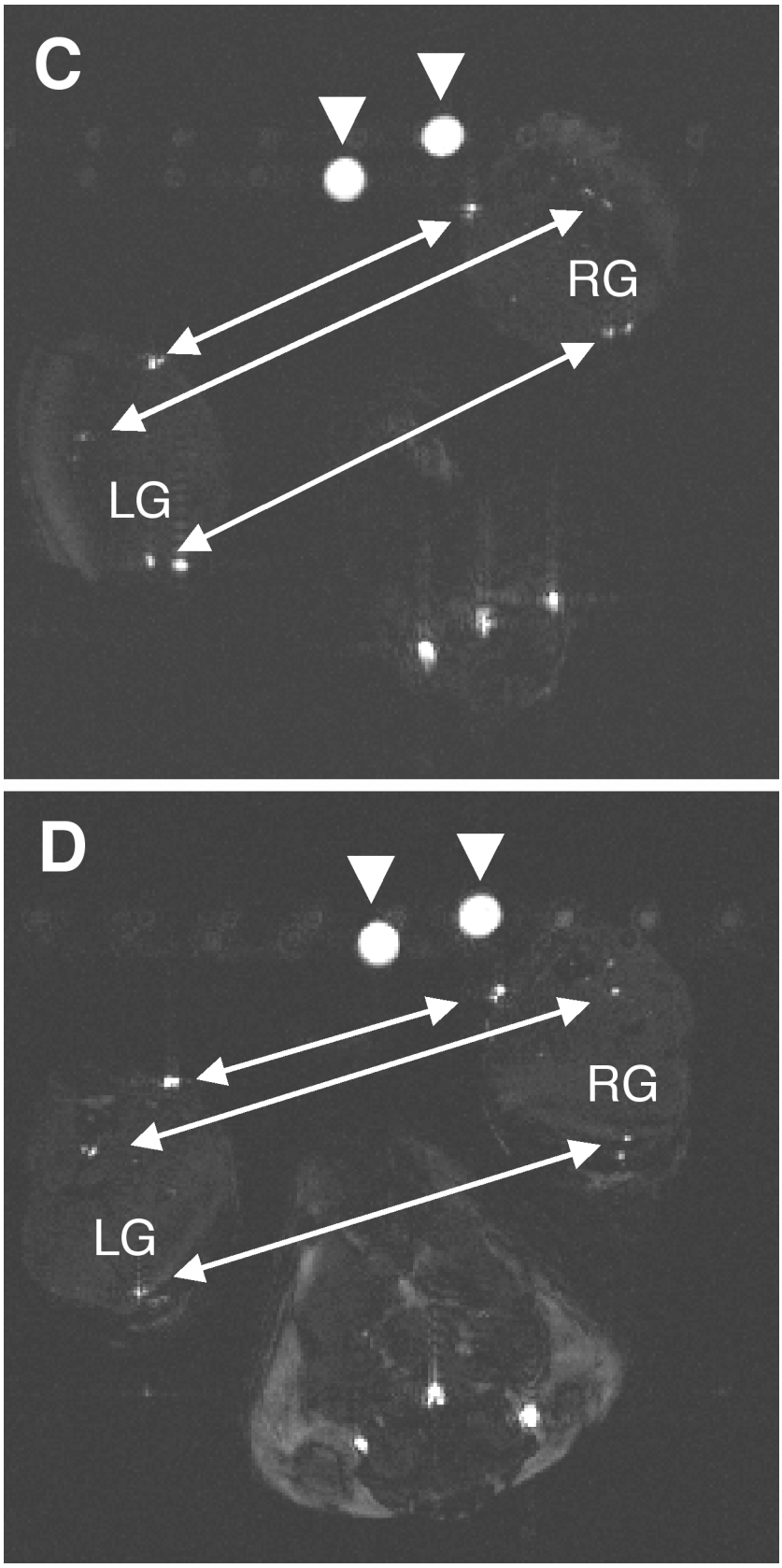


Figure 18. *Representative cross-sectional Magnetic Resonance Images (MRI) of* ***A****) a sedentary nontransgenic mouse,* ***B****) a sedentary FGF–2 transgenic mouse,* ***C****) a trained nontransgenic mouse, and* ***D****) a trained FGF–2 transgenic mouse. Note the three main arteries (arrows) of the gastrocnemius (RG=right gastrocnemius (occluded leg), LG=left gastrocnemius (normal leg)). The tubes for*

determination of the standard flow are marked by the arrowheads. Structures in lower part of the images represent the caudal part of the mouse including the tail artery and veins.

Table 4. Collateral dependent blood flow to the foot determined by Laser Doppler Imaging and to the gastrocnemius determined by Magnetic Resonance Imaging of sedentary nontransgenic, trained nontransgenic, sedentary FGF–2 transgenic, and trained FGF–2 transgenic mice.

Blood Flow	Sedentary Non-Transgenic	Trained Non-Transgenic	Sedentary FGF–2 Transgenic	Trained FGF–2 Transgenic
LDI of Foot (right/left ratio)	0.66 ± 0.10	0.78 ± 0.06	0.88 ± 0.06	0.97 ± 0.03*
MRI of Gastrocnemius (ml/min/g) Occluded leg	0.60 ± 0.06	0.61 ± 0.10	0.85 ± 0.10*	0.85 ± 0.06*
MRI of Gastrocnemius (ml/min/g) Normal leg	0.83 ± 0.06	0.93 ± 0.07	1.00 ± 0.15	1.16 ± 0.11*

Data are means ± SEM. * $p < 0.05$ vs. nontransgenic sedentary mice.

5.3.3. Exercise Capacity

Exercise capacity of trained FGF–2 transgenic mice was significantly increased when compared to trained nontransgenic mice, sedentary FGF–2 transgenic mice, and sedentary nontransgenic mice (139.17 ± 11.12 J vs. 99.08 ± 9.07 J,

50.95 ± 2.73 J, 45.10 ± 4.19 J, respectively, Figure 19, Table 5). Exercise capacity of trained FGF–2 transgenic and trained nontransgenic mice increased by 208% and 120%, respectively, when compared to sedentary nontransgenic mice. There was no statistically significant difference between sedentary transgenic and sedentary nontransgenic mice.

Trained nontransgenic and trained FGF–2 transgenic mice were running without visible limitation by the occluded leg. However, both sedentary groups were impaired in their performance by the occlusion of the right femoral artery. This effect was characterized by limping of the right hindlimb and by making contact with the right wall of the treadmill lane in which the mouse was running.

Table 5. Exercise capacity of sedentary nontransgenic, sedentary FGF–2 transgenic, trained nontransgenic, and trained FGF–2 transgenic mice at day 36 after occlusion of the right femoral artery.

Exercise Capacity Influenced by:	**Genotype: Nontransgenic**	**Genotype: FGF–2 Transgenic**
Activity: Sedentary	45.10 ± 4.19 J	50.95 ± 2.73 J
Activity: Trained	99.08 ± 9.07 J*	139.17 ± 11.12 J**

Data are means ± SEM.* $p < 0.05$ vs. nontransgenic sedentary mice,

** $p < 0.05$ vs. nontransgenic trained mice.

5.3.4. Gastrocnemius Weight-to-Body Weight Ratio

Right gastrocnemius weight-to-body weight ratio of sedentary nontransgenic mice was significantly decreased by 15% when compared to the normal left side (7.19 ± 0.43 mg/g vs. 8.45 ± 0.42 mg/g, respectively, Figure 20, Table 6). Right gastrocnemius weight-to-body weight ratio of trained nontransgenic mice was significantly decreased by 14% when compared to the normal left side (7.37 ± 0.10 mg/g vs. 8.57 ± 0.28 mg/g, respectively).

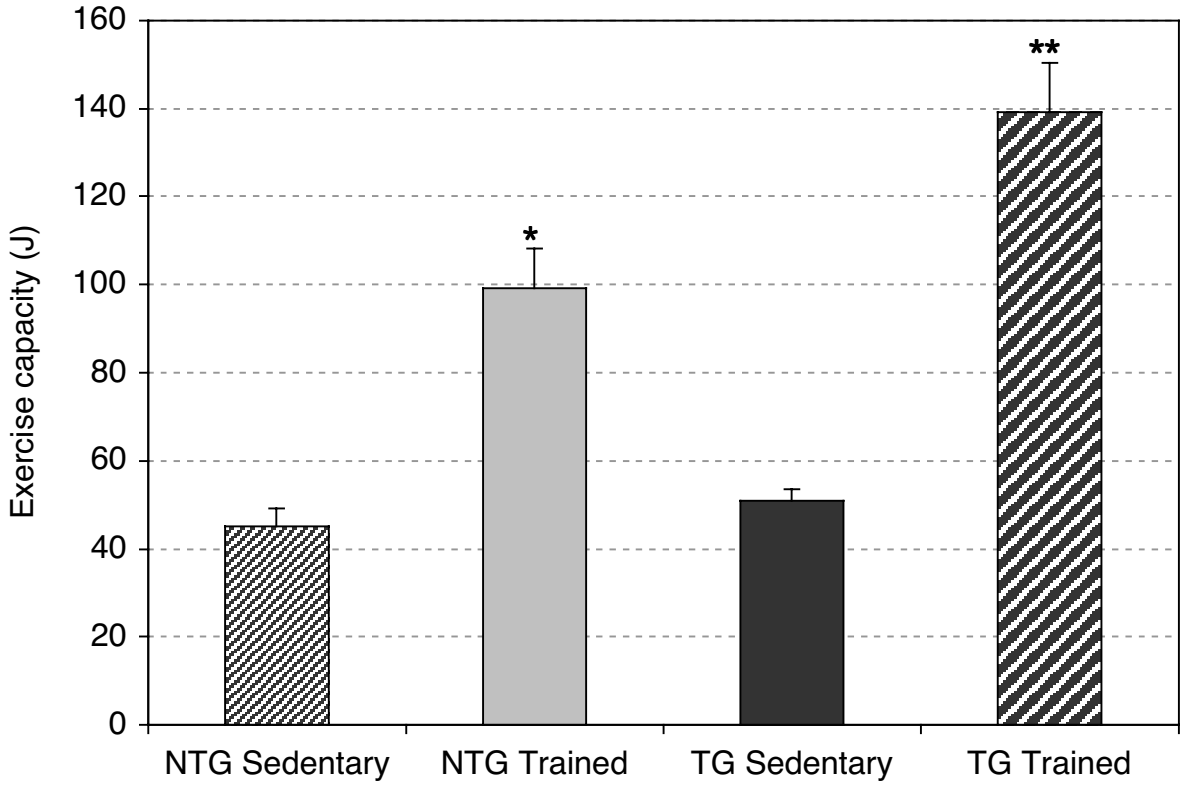


Figure 19. *Exercise capacity of sedentary nontransgenic (NTG Sedentary), trained nontransgenic (NTG Trained), sedentary FGF–2 transgenic (TG Sedentary), and trained FGF–2 transgenic (TG Trained) mice at day 36 after occlusion of the right femoral artery. Data are means ± SEM. * $p < 0.05$ vs. nontransgenic sedentary mice, ** $p < 0.05$ vs. nontransgenic trained mice.*

Table 6. Gastrocnemius-to-body weight ratio of sedentary nontransgenic, trained nontransgenic, sedentary FGF–2 transgenic, and trained FGF–2 transgenic mice.

Gastrocnemius-to-Body Weight Ratio	**Right Gastrocnemius** (mg/g)	**Left Gastrocnemius** (mg/g)
Sedentary Nontransgenic	7.19 ± 0.43*	8.45 ± 0.42
Trained Nontransgenic	7.37 ± 0.10*	8.57 ± 0.28
Sedentary FGF–2 Transgenic	7.34 ± 0.44	7.79 ± 0.33
Trained FGF–2 Transgenic	7.29 ± 0.15	7.93 ± 0.41

Data are means ± SEM. * $p < 0.05$ vs. left gastrocnemius of same group.

There was no statistically significant difference between the right and left gastrocnemius weight-to-body weight ratios of sedentary FGF–2 transgenic mice (7.34 ± 0.44 mg/g vs. 7.79 ± 0.33 mg/g, respectively). Similarly, the right and left gastrocnemius weight-to-body weight ratios of trained FGF–2 transgenic mice were not significantly different (7.29 ± 0.15 mg/g vs. 7.93 ± 0.41 mg/g, respectively). The difference in left gastrocnemius weight-to-body weight ratio between all groups was not statistically significant. However, a trend towards a lower left gastrocnemius weight-to-body weight ratio of the two transgenic groups was noted.

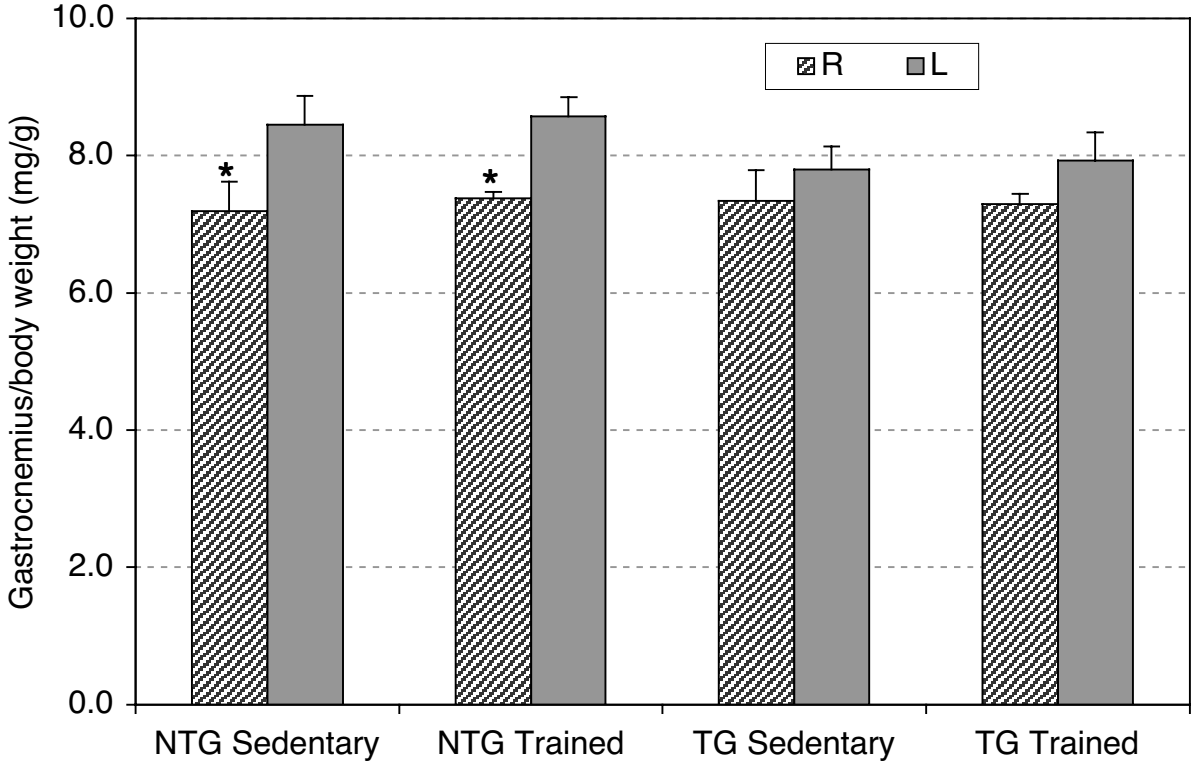
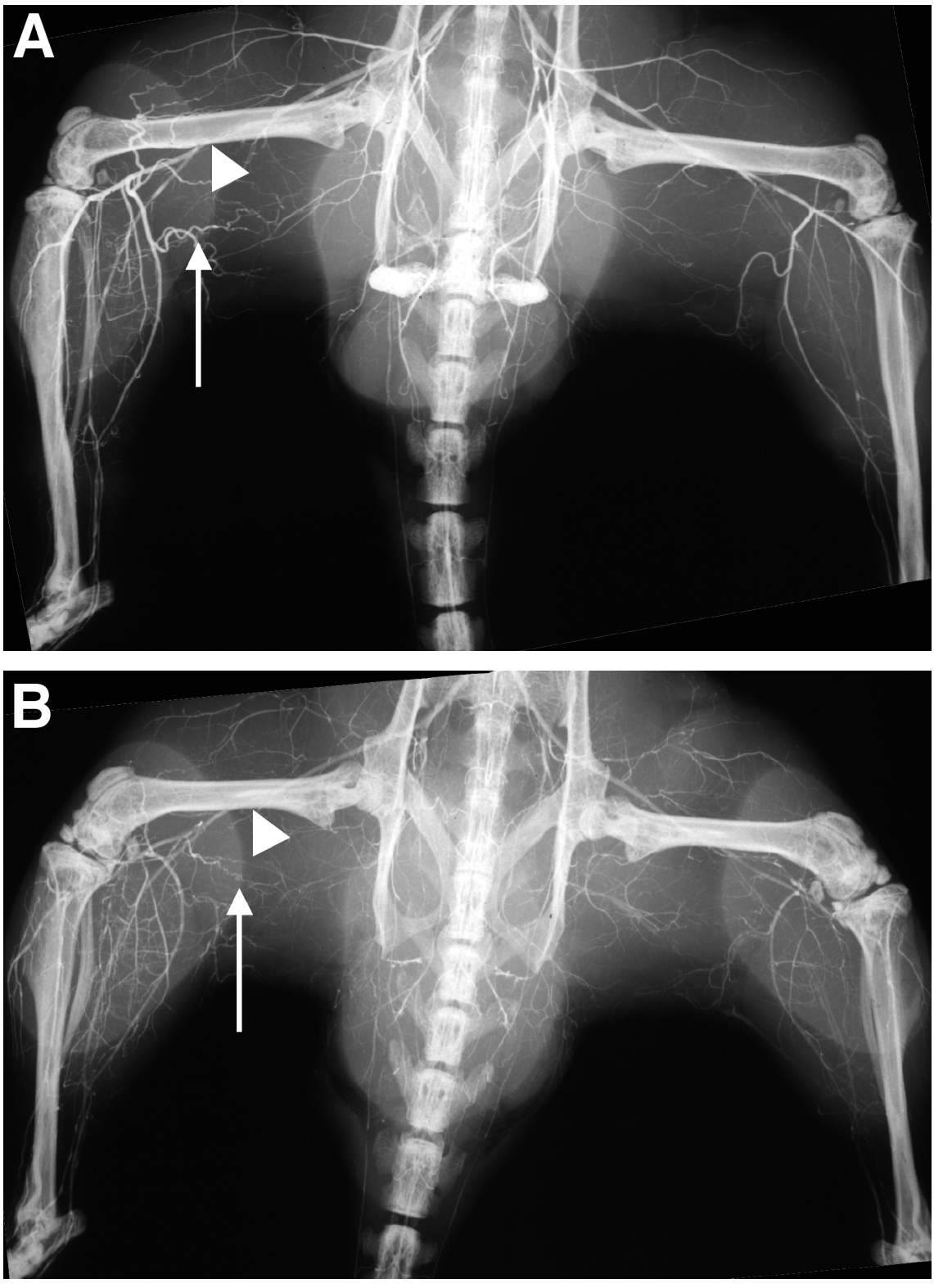


Figure 20. *Gastrocnemius-to-body weight ratio of sedentary nontransgenic (NT Sedentary), trained nontransgenic (NTG Trained), sedentary FGF–2 transgenic (TG Sedentary), and trained FGF–2 transgenic (TG Trained) mice. Data are means ± SEM. * $p < 0.05$ vs. left gastrocnemius of same group*

Postmortem angiographies of sedentary nontransgenic and FGF–2 transgenic mice demonstrated the typical corkscrew growth pattern of collateral arteries (Figure 21). Collateral arteries of sedentary FGF–2 transgenic mice appeared to be slightly bigger than those of sedentary nontransgenic mice. Trained nontransgenic mice displayed more diffuse angiographies with numerous smaller vessels and were lacking the corkscrew growth pattern. Similarly, trained FGF–2 transgenic mice demonstrated a diffuse picture. However, even more

smaller vessels were apparent. These vessels were contributed over the entire proximal hindlimb. Even though the typical corkscrew growth pattern was lacking and the precise origins and ends of these vessels were sometimes difficult to determine, they still appeared to serve as collateral connections passing the site of occlusion.



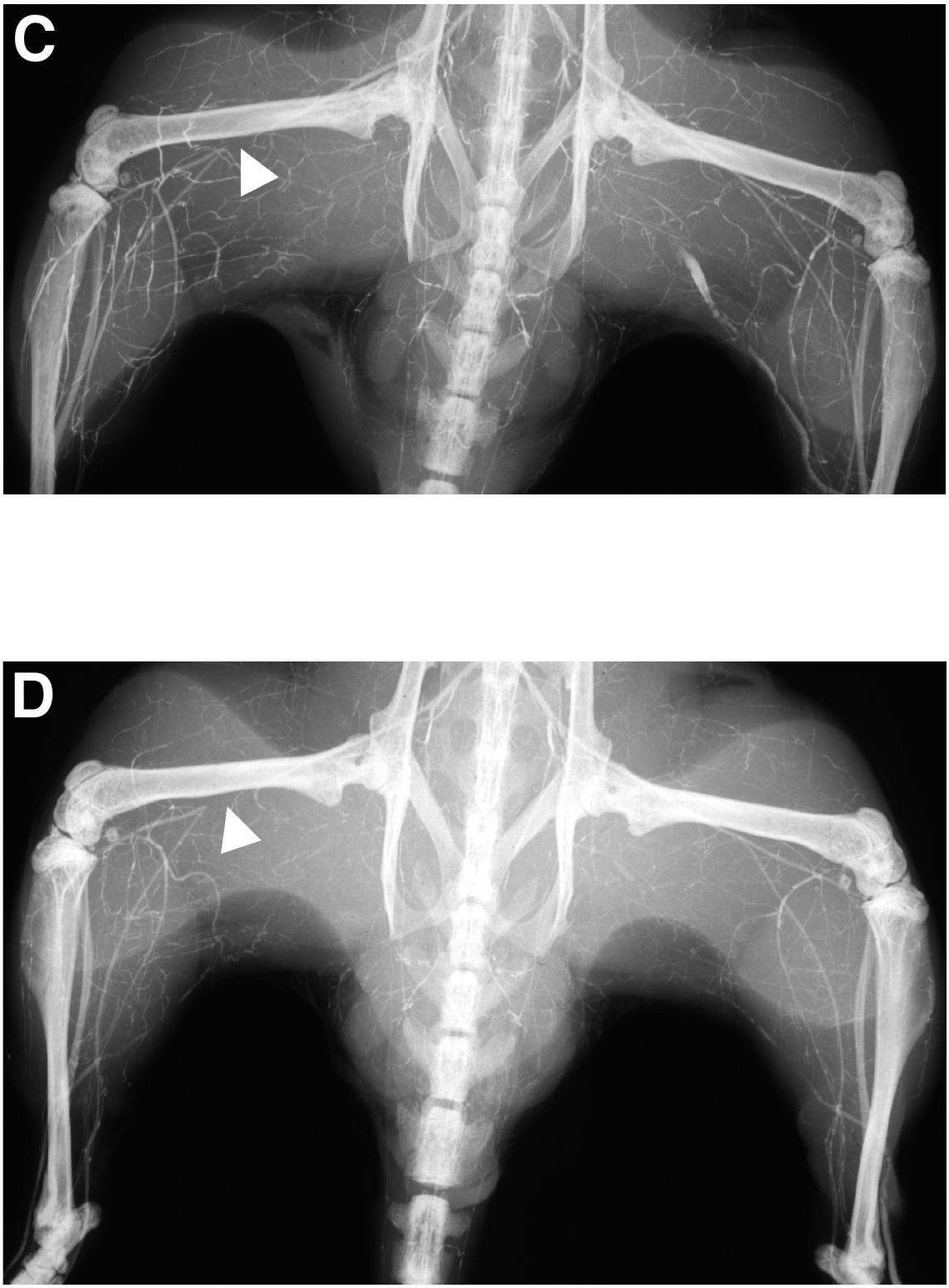


Figure 21. *Representative angiographies of* ***A****) sedentary nontransgenic mice,* ***B****) sedentary FGF–2 transgenic mice,* ***C****) trained nontransgenic mice, and* ***D****) trained FGF–2 transgenic mice. Note the site of occlusion (arrowhead) and the typical corkscrew growth pattern of the collateral arteries in sedentary mice (arrow). Trained nontransgenic mice display a dense collateral network with numerous smaller vessels. Trained FGF–2 transgenic mice show a diffuse growth pattern with the highest vessel density of all groups.*

5.4. Determination of Exercise Capacity

5.4.1. Time Course after the Onset of Congestive Heart Failure

At the age of 16 weeks, exercise capacity of MCP–1 transgenic mice was significantly reduced by 38% when compared to nontransgenic control mice (72.45 ± 5.63 J vs. 117.47 ± 10.00 J, respectively, Figure 22, Table 7). At the age of 20 weeks, the same difference was apparent (36% reduction) with both MCP–1 transgenic and nontransgenic mice displaying approximately the same exercise capacity determined initially (71.55 ± 6.08 J vs. 112.55 ± 16.60 J, respectively). At the age of 24 weeks, exercise capacity of MCP–1 transgenic mice was reduced by 42% when compared to nontransgenic mice (59.12 ± 6.74 J vs. 102.11 ± 7.38 J, respectively). At week 28 exercise capacity of nontransgenic animals was 100.45 ± 8.97 J. Five of the remaining seven MCP–1 transgenic mice had died due to congestive heart failure caused by chronic myocarditis. The two surviving mice displayed symptoms of severe heart failure including dyspnea at rest and massive edema. One transgenic mouse died one minute after initiation of the exercise test. The other transgenic mouse was not able to start running and no attempt was made to submit the mouse to a complete exercise test. After the sudden death of the first mouse and after the observation that the second mouse was not able to perform any work, the thorax' of these mice were opened immediately. Massive ventricular and atrial hypertrophy was observed. The ventricles appeared to be enlarged approximately 2 fold while the atria seemed to be even bigger than that compared to hearts of nontransgenic mice.

Table 7. Exercise capacity of nontransgenic control mice and MCP–1 transgenic mice at the age of 16, 20, 24, and 28 weeks.

Exercise Capacity	Nontransgenic	MCP–1 Transgenic
16 weeks	117.47 ± 10.00 J	72.45 ± 5.63 J*
20 weeks	112.55 ± 16.60 J	71.55 ± 6.08 J*
24 weeks	102.11 ± 7.38 J	59.12 ± 6.74 J*
28 weeks	100.45 ± 8.97 J	/

Data are means ± SEM. * $p < 0.05$ vs. nontransgenic mice.

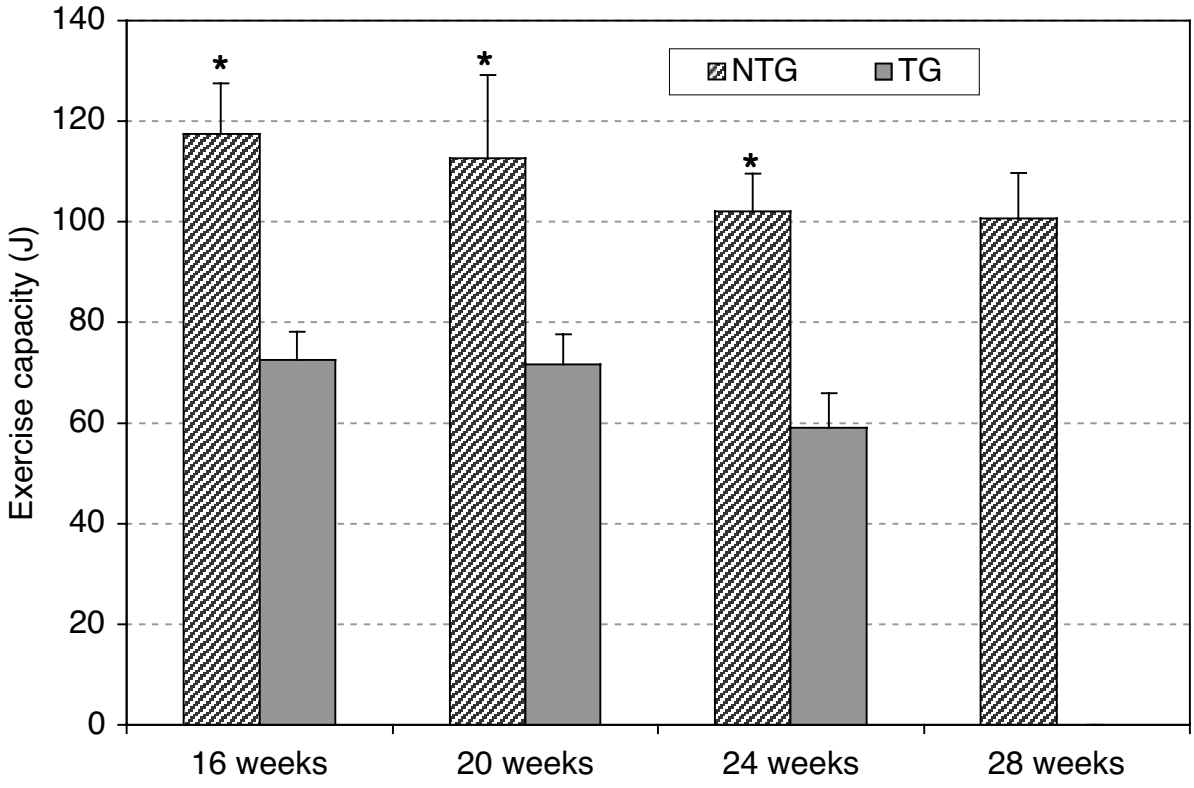


Figure 22. *Exercise capacity of nontransgenic control mice (NTG) and MCP–1 transgenic mice (TG) at the age 16, 20, 24, and 28 weeks. Data are means ± SEM. * $p < 0.05$ vs. nontransgenic mice.*

Both mice had severe ascites and pulmonary congestion. The slight decrease in exercise capacity of nontransgenic mice over the experimental period of 16 weeks was not statistically significant. At each time point tested the MCP–1 transgenic mice exhibited stronger signs of fatigue after the exercise test when compared to nontransgenic animals. A characteristic drop of water forming under the nose of the mouse at the end of the exercise test was observed frequently in MCP–1 transgenic mice. In nontransgenic mice this drop was observed but less frequent. The loss of the righting reflex was found in every transgenic animal and lasted longer than in nontransgenic mice. After the test transgenic mice displayed pronounced dyspnea. The phase of voluntary inactivity after the exercise test was longer than that of nontransgenic mice.

5.4.2. Time Course after Bilateral Femoral Artery Occlusion

Collateral dependent blood flow to the foot determined by LDI revealed a significant increase in blood flow over a period of four weeks post occlusion. The major changes occurred within the first 2 weeks. The next two weeks were characterized by only minor changes in collateral dependent blood flow to the foot. Balb/C mice demonstrated the least increase in foot blood flow after femoral artery occlusion. Right-to-left ratio increased from 0.01 ± 0.01 immediately after occlusion to 0.05 ± 0.01 at day 3, to 0.21 ± 0.05 at day 7, to 0.37 ± 0.06 at day 14, remained unchanged at day 21 (0.36 ± 0.05), and increased to 0.51 ± 0.07 at day 28 (Figure 23, Table 8). Collateral dependent blood flow to the foot of sv129 mice increased significantly more during the study period. Immediately after occlusion right-to-left ratio was already 0.16 ± 0.05 and increased to 0.34 ± 0.10 at day 3, to 0.62 ± 0.09 at day 7, to 0.83 ± 0.09 at day 14, to 0.81 ± 0.09 at day 21, to 0.92 ± 0.12 at day 28 post occlusion. The fastest increase in collateral dependent blood flow to the foot was observed in C57Bl/6 mice. Right to left ratio increased from 0.10 ± 0.03 immediately after occlusion to 0.36 ± 0.08 at day 3, to

Table 8. Collateral dependent blood flow to the foot determined by LDI of Balb/C, sv129, and C57Bl/6 mice with unilateral femoral artery occlusion immediately before and after occlusion, and at days 3, 7, 14, 21, and 28.

Blood Flow (Right/left ratio)	**Balb/C**	**sv129**	**C57Bl/6**
Before	0.98 ± 0.06	0.96 ± 0.02	0.97 ± 0.02
After	0.01 ± 0.01	0.16 ± 0.05	0.10 ± 0.03
Day 3	0.05 ± 0.01	0.34 ± 0.10	0.36 ± 0.08*
Day 7	0.21 ± 0.05	0.62 ± 0.09*	0.73 ± 0.06*
Day 14	0.37 ± 0.06	0.83 ± 0.09*	0.97 ± 0.04*
Day 21	0.36 ± 0.05	0.81 ± 0.09*	1.03 ± 0.04*
Day 28	0.51 ± 0.07	0.92 ± 0.12*	1.02 ± 0.04*

Data are means ± SEM. * $p < 0.05$ vs. Balb/C mice.

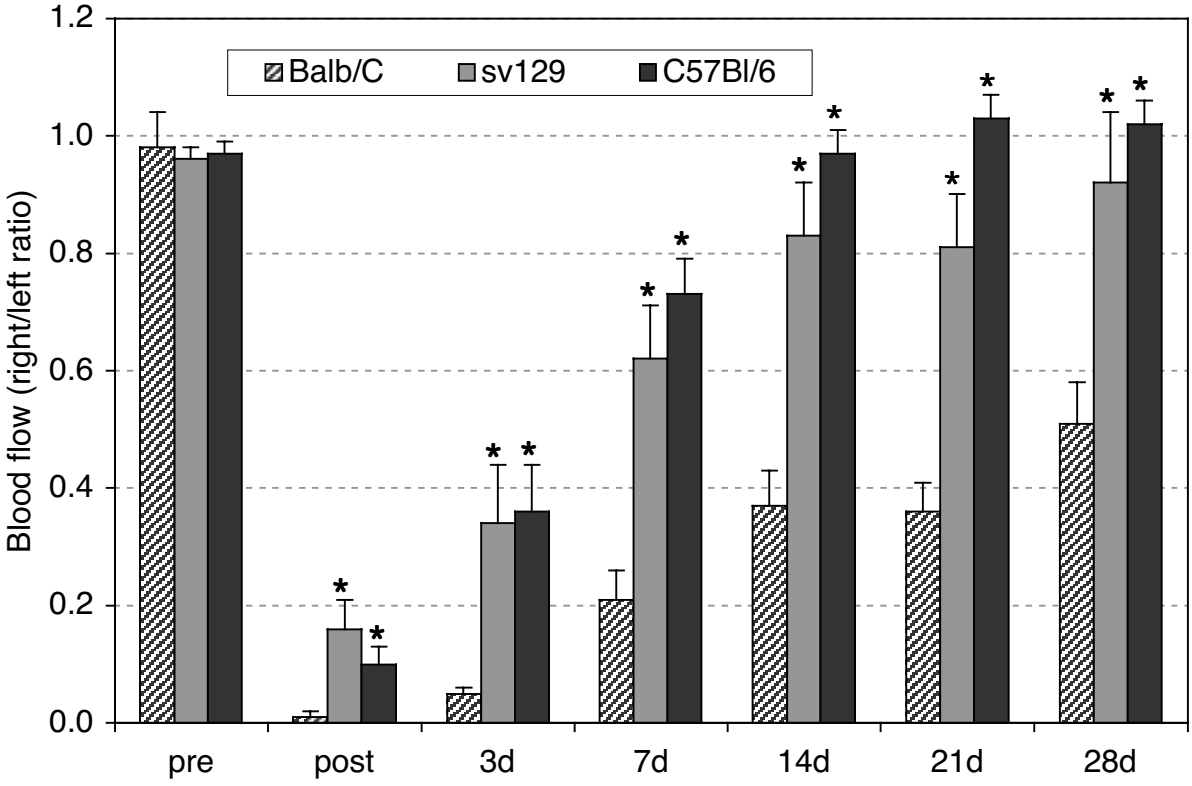


Figure 23. *Collateral dependent blood flow to the foot (right / left ratio) determined by LDI of Balb / C, sv129, and C57Bl / 6 mice with unilateral femoral artery occlusion immediately before and after occlusion and at days 3, 7, 14, 21, and 28. Data are means ± SEM. * $p < 0.05$ vs. Balb / C.*

0.73 ± 0.06 at day 7, to 0.97 ± 0.04 at day 14, and remained unchanged at day 21 (1.03 ± 0.04) and at day 28 (1.02 ± 0.04).

Determination of exercise capacity revealed a significant difference between normal Balb/C (102.91 ± 6.59 J) and sv129 as well as C57Bl/6 mice (72.87 ± 6.49 J and 70.91 ± 3.46 J, respectively, Figure 24, Table 9). At day seven after bilateral femoral artery occlusion there was no significant difference in exercise capacity between Balb/C, sv129 and C57Bl/6 mice (26.13 ± 3.46 J vs. 21.20 ± 1.58 J vs. 23.30 ± 3.23 J, respectively). At day 14 post occlusion the exercise capacities of mice of the three different strains were again similar (42.58 ± 5.97 J vs. 37.03 ± 4.44 J vs. 41.73 ± 4.78 J, respectively). No significant change in exercise capacity could be observed at day 21 after occlusion when comparing Balb/C, sv129 and C57Bl/6 mice (48.20 ± 3.31 J vs. 48.20 ± 4.64 J vs. 45.71 ± 3.95 J, respectively). There was little change in exercise capacity during the fourth week after bilateral femoral artery occlusion and the difference between the three strains still did not reach the level of significance (49.02 ± 3.59 J vs. 48.67 ± 4.74 J vs. 46.38 ± 4.07 J, respectively).

Table 9. Exercise capacity of unoperated Balb/C, sv129, and C57Bl/6 mice and at days 7, 14, 21, and 28 after bilateral femoral artery occlusion.

Exercise Capacity	Balb/C	sv129	C57Bl/6
Control	102.91 ± 6.59 J*	72.87 ± 6.49 J	70.91 ± 3.46 J
Day 7	26.13 ± 3.46 J	21.20 ± 1.58 J	23.30 ± 3.23 J
Day 14	42.58 ± 5.97 J	37.03 ± 4.44 J	41.73 ± 4.78 J
Day 21	48.20 ± 3.31 J	48.20 ± 4.64 J	45.71 ± 3.95 J
Day 28	49.02 ± 3.59 J	48.67 ± 4.74 J	46.38 ± 4.07 J

Data are means ± SEM. * $p < 0.05$ vs. sv129 and C57Bl/6 mice.

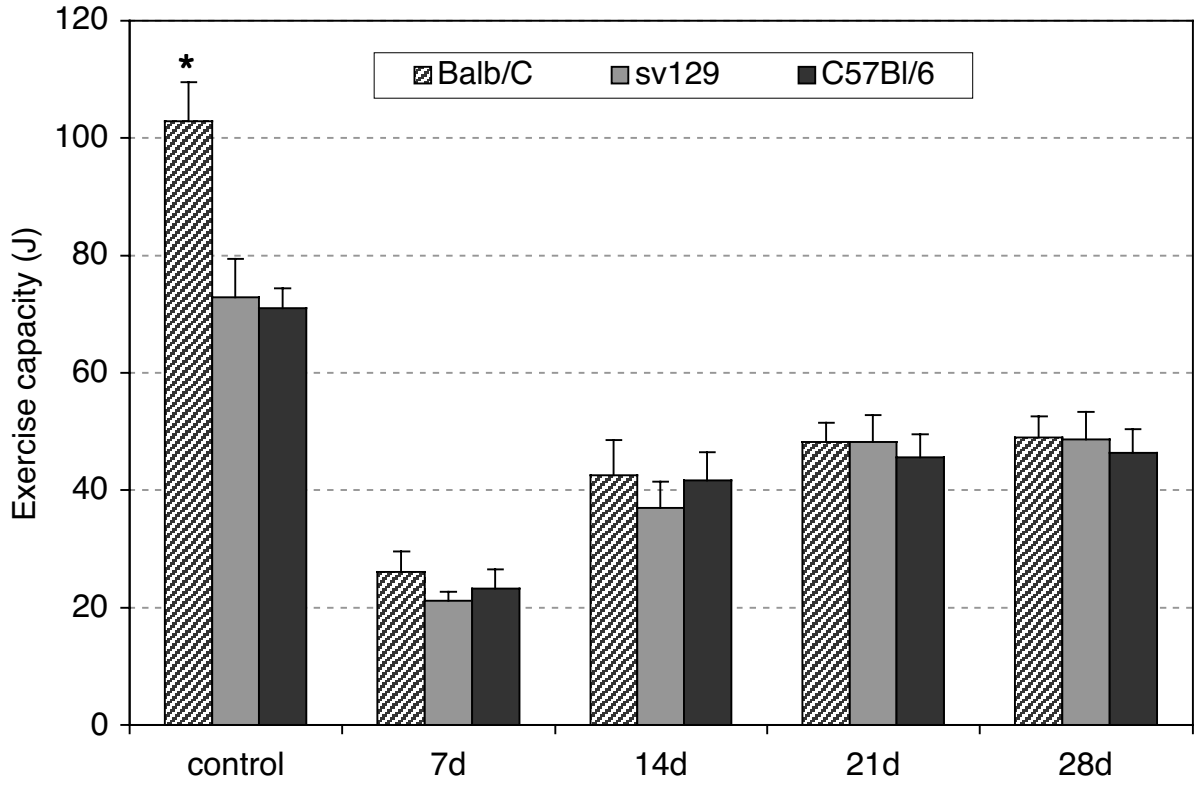


Figure 24. *Exercise capacity of unoperated Balb/C, sv129, and C57Bl/6 mice and at days 7, 14, 21, and 28 after bilateral femoral artery occlusion. Data are means ± SEM. * $p < 0.05$ vs. sv129 and C57Bl/6 mice.*

However, when exercise capacity of mice with bilateral femoral artery occlusion was blotted as the percentage of unoperated control mice significant differences in the recovery of exercise capacity could be observed. When comparing the recovery of exercise capacity of Balb/C, sv129 and C57Bl/6 mice at day seven post occlusion a trend towards an increase was observed (25.39 ± 3.36% vs. 29.09 ± 2.17% vs. 32.86 ± 4.56%, respectively, Figure 25, Table 10). At day 14 this trend became statistically significant for C57Bl/6 mice when compared to Balb/C (58.85 ± 6.74% vs. 41.38 ± 5.80%, respectively). The recovery of exercise capacity of sv129 mice was 50.82 ± 6.09%, which was not significantly different from the two other strains. At day 21 post occlusion a significant difference between Balb/C versus sv129 and C57Bl/6 was observed (46.84 ± 3.22% vs. 66.15 ± 6.37% vs. 64.46 ± 5.57%, respectively). This difference was still apparent at day 28 with only a slight increase in recovery of exercise capacity of each strain (47.63 ± 3.49% vs. 66.79 ± 6.50% vs. 65.41 ± 5.74%, respectively).

Exercise tests at days 7 and 14 post ligation demonstrated a severe limitation of the performance of all mice by the bilateral femoral artery occlusion. The point of fatigue was determined if the animal was only dragging their hindlimbs and not properly moving them. However, exercise tests at days 21 and 28 showed a gain in function of the hindlimbs at the end of the test. The limitation of performance could not exclusively be ascribed to femoral artery occlusion. When compared to control mice there was still a difference in the behavior on the treadmill especially at the end of the test.

Table 10. Recovery (% of control mice) of exercise capacity of Balb/C, sv129, and C57Bl/6 mice with bilateral femoral artery occlusion at days 7, 14, 21, and 28.

Exercise Capacity	Balb/C	sv129	C57Bl/6
Control	100.00 ± 6.40%	100.00 ± 8.91%	100.00 ± 4.88%
Day 7	25.39 ± 3.36%	29.09 ± 2.17%	32.86 ± 4.56%
Day 14	41.38 ± 5.80%	50.82 ± 6.09%	58.85 ± 6.74%*
Day 21	46.84 ± 3.22%	66.15 ± 6.37%*	64.46 ± 5.57%*
Day 28	47.63 ± 3.49%	66.79 ± 6.50%*	65.41 ± 5.74%*

Data are means ± SEM. * $p < 0.05$ vs. Balb/C mice.

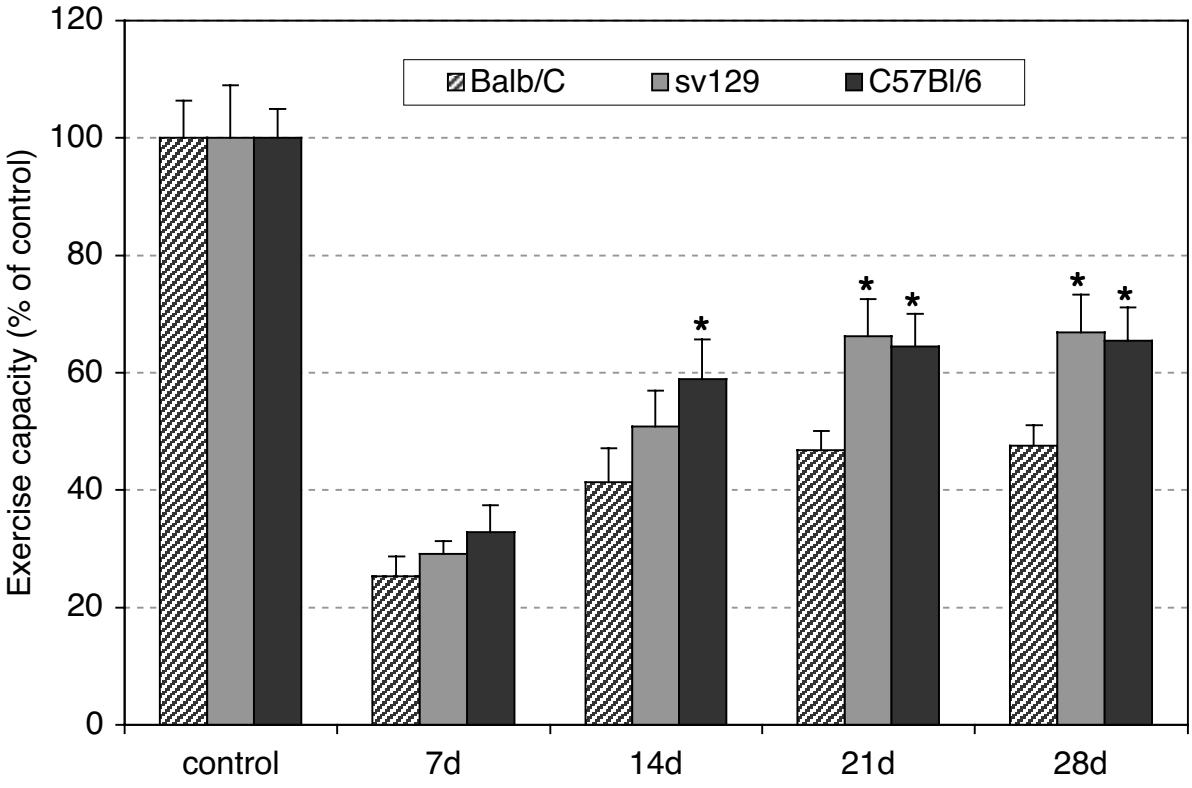


Figure 25. *Recovery (% of control mice) of exercise capacity of Balb/C, sv129, and C57Bl/6 mice with bilateral femoral artery occlusion at days 7, 14, 21, and 28 post occlusion. Data are means ± SEM. * $p < 0.05$ vs. Balb/C mice.*

6. Discussion

6.1. Simulated Ischemia

A study conducted in our laboratory [64] demonstrated that mice with cardiac-specific overexpression of FGF–1 display a marked cardioprotection when submitted to myocardial infarction induced by ligation of the left coronary artery (see also chapter 3.1.5.). However, infarct development was only delayed and not prevented by overexpression of FGF–1. This suggested that a direct protective effect of FGF–1 on cardiac myocytes was responsible for these findings rather than the increase in the density of small arterioles demonstrated in another study [48]. There exists a direct relation between the amount of permanently salvaged myocardium and collateral flow. Since permanent salvage was not achieved we concluded that collateral vessels did not contribute to the increased arteriolar density. However, to undoubtedly prove that FGF–1 overexpression increased the tolerance of cardiac myocytes towards ischemia we conducted the experiments of simulated ischemia.

When isolated cardiac myocytes were submitted to simulated ischemia, cell viability of transgenic cardiac myocytes was increased at the first two of three time points. CK and LDH are two commonly used enzymes and their release from cells is indicative of cell death. Similar to our *in vivo* study, the cardioprotective effect of FGF–1 occured during the early time points. At 4 hours of simulated ischemia CK release from nontransgenic control myocytes was increased approximately 10 fold when compared to the CK release under normoxic conditions during the 16 hours of the experimental protocol. Transgenic myocytes on the other hand increased the CK release only 4 fold, indicating a markedly enhanced viability of transgenic myocytes. This effect was still apparent at 8 hours of simulated ischemia. However, the protective effect of FGF–1 overexpression on cardiac myocyte viability was abolished at 12 hours of simulated ischemia. The release of CK of nontransgenic and transgenic cardiac myocytes was similar at 12 hours of simulated ischemia.

When LDH was used as a marker of cell death, similar results were obtained. Interestingly, transgenic cardiac myocytes did not release more LDH at 4 hours of simulated ischemia when compared to the basal release under normoxic conditions during the 16 hours of the experimental period. This suggests that 4 hours of simulated ischemia caused very little damage. However, the increased CK release at 4 hours compared to the basal release demonstrates that it would be an underestimation to state that 4 hours of simulated ischemia did not cause

any cell damage. Nontransgenic control myocytes increased the LDH release at the same time point by approximately 60%, showing a clear increase in cell death. At 8 hours of simulated ischemia nontransgenic control myocytes displayed significantly increased LDH release when compared to transgenic myocytes. The difference between the LDH release of nontransgenic and transgenic myocytes was smaller but still statistically significant. Again, at 12 hours of simulated ischemia, no difference in LDH release between nontransgenic and transgenic cardiac myocytes was observed, indicating a loss of the cardioprotective effect at later time points.

Both markers of cell death revealed a strong cardioprotective effect of FGF–1 overexpression on isolated ventricular cardiac myocytes. The effect was most pronounced at the earliest time point of simulated ischemia. The protective effect decreased at 8 hours of simulated ischemia but was still prominent and statistically significant. However, FGF–1 overexpression could only delay but not abolish the damaging effects of simulated ischemia as indicated by similar release of cell death markers at 12 hours.

These *in vitro* findings closely resemble the situation found *in vivo*. Therefore, we conclude that cardiac-specific overexpression of FGF–1 delays infarct development *in vivo* and increases the tolerance of isolated cardiac myocytes towards simulated ischemia. The results of the *in vitro* experiments demonstrate that the delayed infarct development was caused by cardiac myocytes, which display enhanced viability under ischemic conditions. We could show with these experiments that the increased density of small arteries and arterioles did not provide any protective effect and thus, was not the reason for the observed delay in infarct development.

Our findings are in accordance with a recently published study that investigated the cardioprotective effect of FGF–2 on hearts of mice overexpressing FGF–2 [237]. Similar to our model of cardiac-specific overexpression of FGF–1 no significant effect on heart weight-to-body weight ratio was noted. FGF–2 overexpression resulted in a 20% increase in capillary density. Thus, FGF–1 and FGF–2 exert different effects considering the growth of capillaries and of arterioles and small arteries. The investigators utilized an isolated mouse heart model of ischemia-reperfusion to access the cardioprotective effect of FGF–2 overexpression. The LDH release of transgenic and nontransgenic mouse hearts was determined in the perfusate. A significantly decreased release from transgenic hearts was found during the reperfusion period, indicating a strong cardioprotective effect of FGF–2. However, this study accessed only the cardioprotective effect of FGF–2

during 30 minutes of ischemia followed by 60 minutes of reperfusion. Investigating more time points and evaluating the total release of LDH during the reperfusion period could have revealed more information. Nevertheless, it is evident that cardiac-specific overexpression of FGF–1 as well as FGF–2 exert strong cardioprotective effects.

The aforementioned results proved our initial hypothesis and were therefore very valuable. However, this study revealed other interesting details that were previously not described in the literature. The basal release of LDH for example, was higher when compared to the basal release of CK. If taken as the only marker, one would think that, in the case of CK, only approximately 3% of the cells die under normoxic conditions during the 16 hours of the experimental protocol. However, under the same conditions the basal release of LDH was much higher when compared to the release of CK. The LDH release of approximately 35% under normoxic condition during the 16 hours of the experimental protocol suggests that about one third of all cardiac myocytes die during the control period.

Frequent microscopic evaluation of the dishes revealed that the basal LDH release most likely reflects the actual number of dead cells. After 12 hours of anoxia, only a fairly small percentage of myocytes was still rod-shaped and had intact cell membranes. Again, LDH release appeared to reflect the situation better than CK release.

The fact that approximately one third of all myocytes dies under normoxic conditions during the 16 hours of the experimental period is easy to explain and no surprise. Adult ventricular cardiac myocytes are extremely fragile and sensitive cells that have very stringent requirements on culture media, temperature, oxygen tension, etc.. Since the used medium lacks any growth factors and no FCS was added, the cardiac myocytes were not kept under optimal conditions. Therefore, it is not surprising that approximately one third of all myocytes died within the control period.

The observation that the release of CK and LDH from isolated ventricular cardiac myocytes submitted to the same experimental condition differs, has not been described before. This suggests that the release of CK displays a different kinetic. The cause of this difference has to be clarified. Differences in molecular weight and different structural features might be of importance but this remains a matter of speculation until further studies are conducted. However, these observations are of importance for following studies and for comparisons of different studies using these two markers of cell damage and death.

In conclusion, the experiments of simulated ischemia demonstrated that ventricular cardiac myocytes isolated from adult mice with cardiac specific-overexpression of FGF–1 show a marked delay in cell death when compared to nontransgenic control myocytes. This suggests that overexpression of FGF–1 exerts a cardioprotective effect. However, the cardioprotection delays but does not abolish cell death due to simulated ischemia. These experiments provided an explanation for the *in vivo* finding of a delayed infarct development in transgenic mice. The marker that most accurately reflected the microscopic evaluation of the dishes was the release of LDH. The difference between the release of LDH and CK is pronounced and requires careful interpretation of studies using either one of these markers.

6.2. Determination of a Flow-Pressure-Relationship

Investigating the effects of cardiac-specific FGF–1 overexpression on capillary and arterial growth in the heart [48], we found an increase in the number of arterioles and small arteries. Furthermore, the number of branches of the main coronary arteries was significantly increased in transgenic animals. There was no change in the number of capillaries. We hypothesized that the increased number of arterioles would lead to an increase in coronary flow of intact transgenic mouse hearts. Therefore, we accessed the coronary flow by *ex vivo* retrograde perfusion of the heart under maximal vasodilatory conditions at four different pressures.

The established flow-pressure-relationship demonstrated that the increase in arteriolar density is reflected by an enhanced myocardial perfusion. Furthermore, the difference in coronary flow between transgenic and nontransgenic mice increased with the perfusion pressure, resulting in a significant pressure-dependent increase in coronary flow of transgenic mice. This suggests that the increase in the arteriolar density could have important physiological consequences. However, the significance of an enhanced coronary flow, especially at higher pressures, in the *in vivo* situation remains to be determined.

The determination of the flow-pressure-relationship of mice overexpressing FGF–1 revealed that increased branching of the main coronary arteries and an increase in the number of arterioles and small arteries is capable of changing the myocardial perfusion.

The isolated heart preparation for determination of the coronary flow in order to reflect vascular growth was recently used by another group of investigators [238]. The pressure-flow relationship was established in a slightly different manner than in our experiments. Instead of using different perfusion pressures and monitoring the amount of coronary effluent, these investigators controlled the coronary inflow rate and monitored the required pressure to achieve a certain coronary flow. The angiogenic effect of PR39, a 39 amino acid peptide secreted by macrophages, was reflected by a shift to the left of the pressure-flow curve. The findings that an increase in the number of vessels increases the coronary flow are similar to our results even tough they were obtained with slightly different methods.

However, the linear relationship between the applied pressure and the resulting coronary flow was not accessed in the aforementioned study. Our study revealed that the increase in the number of resistance vessels in the hearts of transgenic mice was reflected by a steeper inclination of the pressure-flow curve. Therefore, we observed at higher pressures a greater difference in coronary flow between transgenic and nontransgenic mouse hearts.

It appears that our model of investigating the coronary flow via establishing a pressure-flow relationship is accurate and suitable for determination of the functional relevance of an increase in the number of small arteries and arterioles in hearts overexpressing FGF–1. The pressure-flow curves reveal important details about the dynamic change of the coronary flow in response to increased perfusion pressures. Therefore, the established protocol is valuable for future studies investigating changes of the coronary vasculature.

6.3. FGF–2 Transgenic Mice with Unilateral Femoral Artery Occlusion

The progressive, moderate intensity endurance exercise program was well tolerated by all mice and induced significant morphological, hemodynamic, and functional changes in trained mice. These changes were more pronounced in mice with a general overexpression of FGF–2. This study revealed interesting details about the functional significance of the exercise program on one hand and of FGF–2 overexpression on the other hand.

Sedentary mice overexpressing FGF–2 increased the hindlimb perfusion of the occluded leg determined by MRI compared to nontransgenic sedentary control

mice. The normal leg displayed slightly (not statistically significant) elevated blood flow. The increase in right-to-left ratio determined by LDI did not achieve the level of significance. Thus, the increase in hindlimb blood flow due to FGF–2 overexpression in sedentary mice was at the level of borderline significance.

These findings were reflected by the exercise capacity. Sedentary FGF–2 transgenic mice displayed a small increase in exercise capacity that did not achieve the level of significance when compared to sedentary nontransgenic mice. It would be interesting to see whether the difference in exercise capacity between transgenic and nontransgenic mice would be more pronounced if determined in mice with bilateral femoral artery occlusion. Possibly, the limiting effect of bilateral femoral artery occlusion would be so severe that the small increase in blood flow in transgenic mice would lead to a significant increase in exercise capacity. However, this remains a matter of speculation until clarifying experiments are carried out.

Surprisingly, nontransgenic mice with unilateral femoral artery occlusion that were submitted to the progressive, moderate intensity endurance exercise program increased their exercise capacity by approximately 120% compared to their sedentary counterparts. Hindlimb perfusion of trained nontransgenic mice determined by two different methods was insignificantly less when compared to sedentary transgenic mice. However, since the difference in exercise capacity between the two sedentary groups was small, trained nontransgenic animals increased their exercise capacity also by about 100% when compared to sedentary transgenic mice.

Thus, a fairly small increase in hindlimb perfusion of sedentary FGF–2 transgenic mice lead to a similar, insignificant increase in exercise capacity. However, blood flow levels between that of sedentary nontransgenic and transgenic mice were not reflected by exercise capacity in trained nontransgenic mice. The training program was causing a significant increase in exercise capacity without exerting a major effect on resting hindlimb blood flow.

There was a significant increase in hindlimb blood flow determined by LDI between trained nontransgenic and transgenic mice. However, determination of blood flow by MRI did not reveal significant differences. Therefore, we considered the blood flow difference between the two trained groups to be at the level of borderline significance. However, determination of exercise capacity uncovered the significance of increase in blood flow due to overexpression of FGF–2. Trained transgenic mice displayed a significant 40% increase in exercise capacity when compared to trained nontransgenic mice. When compared to

sedentary nontransgenic mice, trained FGF–2 transgenic mice increased exercise capacity by approximately 200%.

One could speculate that the significant difference in exercise capacity between the two trained groups arises from adaptations due to the endurance exercise program. The increased exercise capacity compared to the sedentary groups can in part be explained by the full range of metabolic alterations due to training (for review see [100]). With the advanced skeletal muscle metabolism during exercise the enhanced blood supply becomes a limiting factor. Then, trained transgenic mice have the advantage of a better blood flow, which is now responsible for a significant difference in exercise capacity.

Another contributing factor to the increased exercise capacity could be an altered pattern of vascular growth. Angiographic observations showed that trained mice form a dense collateral network. The typical corkscrew collateral arteries developed only in sedentary mice. It is possible that trained mice have developed a skeletal muscle vasculature that efficiently supplies blood to the whole hindlimb especially during exercise. It seems that the corkscrew growth pattern is capable of increasing calf blood flow and foot blood flow during resting conditions to a certain extent so that no ischemia is present. However, this corkscrew growth pattern might not be sufficient to meet the blood flow needs during exercise, especially of the muscles of the thigh.

Currently, we perform histological studies to precisely determine the vascular growth pattern. We investigate skeletal muscle tissue from the proximal hindlimb where collateral arteries developed (arteriogenesis) and from the distal hindlimb where possibly angiogenesis occurred. Analysis of these skeletal muscle samples will provide us with information whether the increased vessel density observed in the angiograms is due to growth of the preexistent collateral vessels in diameter or due to increased arteriolar development and growth. We will determine the extent of angiogenesis in the gastrocnemius and we will investigate the roles of overexpression of FGF–2 and of the endurance exercise program with possible treatment interactions.

It would be of great interest to determine hindlimb blood flow during exercise. Unfortunately, until now we did not succeed to develop a precise system for determination of exercise blood flow. However, similar studies of Terjung and colleagues provide valuable information.

In 1998, they conducted a study in rats investigating the effect of intra arterial FGF–2 infusion via osmotic minipumps [185]. Subgroups of the FGF–2 group and

the carrier group were trained for four weeks twice daily until the point of fatigue was reached. They determined the hindlimb blood flow at the end of contractility tests. Therefore, blood flow measurements can be considered to reflect exercise blood flow. Similar to our study, a stepwise increase in total hindlimb blood flow from the sedentary carrier group to the trained carrier group to the sedentary FGF–2 group and to the trained FGF–2 group was found. The total hindlimb blood flow of the trained FGF–2 group was significantly greater than all other groups. Proximal hindlimb blood flow showed similar results. Distal blood flow of the trained carrier group, sedentary FGF–2 group, and trained FGF–2 group were significantly increased compared to the sedentary carrier group. Tension development of the calf muscles showed similar results compared to our exercise capacity results. Even though blood flow of the trained carrier group was (insignificantly) lower than blood flow of the sedentary FGF–2 group the opposite was true for the developed tension. Similar to our results, muscle performance of the trained FGF–2 group was by far the greatest. Therefore, one could speculate that blood flow levels during exercise in our model would be similar to the resting blood flow levels possibly with increased absolute values and greater differences between the groups. The order, however, would probably stay the same.

Our study demonstrated that trained mice generally overexpressing FGF–2 display an increased exercise capacity when compared to all other groups. This finding opposes the results reported in the aforementioned study [185]. In this study no significant difference in total running time was observed. However, for determination of exercise capacity we did not just record total running time of training sessions but performed a precise and reproducible exercise test. Furthermore, contractile performance of trained rats with FGF–2 infusion was significantly better when compared to all other groups. It's hard to imagine that the improved contractile performance together with the increased blood flow would not lead to increased exercise capacity under controlled conditions as used in our study.

That trained animals display a significantly increased skeletal muscle blood flow during exercise is shown by another recent study [187]. Rats with prior training to bilateral femoral artery occlusion show a markedly increased skeletal muscle blood flow during exercise when compared to sedentary control rats. A previous study of the same investigators [177] indicates that rats with bilateral femoral artery occlusion, which were then trained for up to eight weeks, increase hindlimb blood flow during exercise significantly when compared to sedentary control animals. Unfortunately, these studies did not determine resting blood flow levels because the investigators did probably not expect any difference.

The current understanding is that exercise uncovers latent ischemia in the heart as well as in limbs. Nevertheless, our resting blood flow measurements determined by MRI and LDI do show differences. However, only the trained FGF–2 transgenic mice demonstrated a significant increase in blood flow determined by both methods. The magnitude of the differences between the other groups is smaller. Statistically significant results are only observed by one of the methods or not at all. Therefore, we can only speculate that resting blood flow measurements reflect to some extent the predicted exercise blood flow measurements. Furthermore, blood flow levels during exercise would probably give rise to a greater difference between the groups.

As described in chapter 5.3.3., the occluded leg of trained mice did not display any functional limitation during the test. This indicated that the blood supply of the occluded hindlimb during exercise was fairly normal and/or that metabolic adaptations developed leading to proper function despite a limited blood supply. Interestingly, FGF–2 overexpression abolished gastrocnemius atrophy due to occlusion of the femoral artery. A significant difference between left and right gastrocnemius weight-to-body weight ratio was only observed in nontransgenic mice. Endurance exercise did not influence the gastrocnemius weight-to-body weight ratios. The atrophy of the right gastrocnemius could have been a limiting factor for exercise capacity of trained nontransgenic animals, leading to a significant decrease when compared to the exercise capacity of trained FGF–2 transgenic mice.

These findings confirm one of the earliest studies investigating the effect of endurance exercise on development of the collateral circulation [97]. Among other results (see also chapter 3.3.) the study demonstrated that femoral artery occlusion causes atrophy of the distal muscles, which is not influenced by an endurance exercise program alone. Many years later, we could now show that overexpression of FGF–2 abolishes distal skeletal muscle atrophy due to femoral artery occlusion. The protective effect of FGF–2 on cells submitted to ischemia [237] could be one of the reasons for this effect. Other explanations involve the angiogenic effect of FGF–2. Better capillary supply of the individual skeletal muscle fibers of the distal hindlimb in FGF–2 transgenic mice could lead to less ischemia and thus, less atrophy.

The slightly decreased gastrocnemius weight-to-body weight ratio of the normal leg of transgenic mice is most likely due to the reduction in bone length caused by overexpression of FGF–2 [228].

Preliminary experiments performed in our laboratory could aid in understanding the mechanism by which trained transgenic animals were able to increase skeletal muscle blood flow as well as exercise capacity significantly. Our initial hypothesis was that training of FGF–2 transgenic mice would lead to a promoter-dependent increase in expression of the transgene. The promoter of phosphoglycerate kinase was used for construction of the transgene. Since this enzyme is a part of glycolysis, we hypothesized that acceleration of glycolysis due to exercise would in turn increase the expression of FGF–2. Furthermore, the phosphoglycerate kinase gene contains a binding element for HIF–1 in its promoter region [239] and HIF–1 expression has been shown to increase in response to exercise [111].

However, preliminary results obtained by Northern blots of phosphoglycerate kinase 1 do not support our initial hypothesis. We did not observe a clear increase in phosphoglycerate kinase 1 expression due to exercise. Nevertheless, we could see increased levels of phosphoglycerate kinase in transgenic animals. The expression patterns between nontransgenic and FGF–2 transgenic mice were also different. The significance of these findings remains to be determined. We currently perform Northern blots of FGF–2 to investigate whether the expression levels of phosphoglycerate kinase and FGF–2 correlate. Furthermore, we will study FGF–2 at the protein level by Western blot analysis. The enhanced release of intracellular FGF–2 in response to stress (exercise) could have increased the amount of FGF–2 in the extracellular space. Therefore, it is possible that trained FGF–2 transgenic mice display enhanced vascular growth due to the increased availability of FGF–2 in the extracellular space.

However, other mechanisms could have also contributed to the observed increase in blood flow and exercise in trained FGF–2 transgenic mice. Exercise for example could have increased shear stress in the collateral circulation, simply providing an additional stimulus for arteriogenesis in the FGF–2 transgenic mice. Another possible explanation could be that overexpression of FGF–2 could have multiplied the growth factor response to exercise (see also chapters 3.1. and 3.3.).

In conclusion, our study demonstrates that trained mice with unilateral femoral artery occlusion overexpressing FGF–2 display increased hindlimb blood flow and exercise capacity. FGF–2 abolished the gastrocnemius atrophy due to femoral artery occlusion. Training did not influence gastrocnemius atrophy. Angiographic observations show the formation of a dense vascular network in trained FGF–2 transgenic mice. Future studies will uncover the mechanism of

this treatment interaction and reveal histological details about the changed vascular growth pattern in trained FGF–2 transgenic mice.

6.4. Determination of Exercise Capacity

6.4.1. Time Course After the Onset of Congestive Heart Failure

Mice with cardiac specific overexpression of MCP–1 displayed already at an age of 16 weeks moderate exercise intolerance as one of the main symptoms of heart failure patients. At an age of 20 weeks exercise capacity remained reduced by the same magnitude when compared to nontransgenic mice. At 24 weeks of age exercise capacity of MCP–1 transgenic mice was decreased by 42%, which was an additional reduction of 6% compared to the month before.

However, during this period of time no major change in exercise capacity occurred. Unexpectedly, signs of exercise intolerance were already found at an age of 16 weeks. The presence of this major symptom of heart failure indicates that deleterious cardiac changes had already occurred. The lack of other symptoms like visible congestion and resting dyspnea suggests that heart failure was at a compensated stage.

During the next eight weeks no major changes in exercise capacity were noted indicating that progression of heart failure was slowly. However, between weeks 24 and 28 of age, five of the seven remaining mice died due to heart failure. Symptoms then included severe congestion and dyspnea at rest. These findings demonstrate progression of heart failure from the compensated to the decompensated stage. The two remaining mice were severely impaired in their ability to perform work. They also displayed severe congestion and resting dyspnea as signs of end-stage congestive heart failure. The facts that one mouse died during the test and that the other remaining mouse could not even start the test illustrate the severity and the progression of heart failure between weeks 24 and 28.

The observed ventricular and atrial hypertrophy was massive and developed mainly during the last weeks of the study period. Hearts taken at the beginning of the study and at an age of 20 weeks did not show signs of hypertrophy, while the heart taken at 24 weeks of age was already moderately hypertrophied.

These observations demonstrate that cardiac-specific overexpression of MCP–1 leads to chronic myocarditis [94] resulting in the development of congestive heart failure. At an age of 16 weeks, exercise intolerance was present as the first sign of heart failure. Thereafter, a slow progression of heart failure was noted without severe worsening of exercise intolerance. Between 24 and 28 weeks of age, cardiac-specific overexpression of MCP–1 led to a sudden and homogeneous switch from the compensated to the decompensated heart failure stage. This resulted first in visible congestion, resting dyspnea, and severe exercise intolerance and later in death of the animals. Mice with decompensated heart failure showed ascites, pulmonary congestion, and massive ventricular and atrial hypertrophy.

Histological examination of the hearts taken at each time point of the study is currently performed in our laboratory and will reveal the degrees of myocarditis, monocyte infiltration, fibrosis, and hypertrophy of ventricular and atrial cardiac myocytes.

Another interesting experiment to perform in the future would be to study the effect of different endurance exercise programs on the development of CHF. Low, moderate, and high intensity endurance exercise programs exert different kinds of stress on the heart. The model of cardiac-specific overexpression of MCP–1 mimics closely the human form of CHF due to chronic myocarditis. Therefore, this study could reveal important information for human patients considering the effect of exercise on progression or regression of CHF. A low intensity endurance exercise program could hypothetically exert a beneficial influence on myocarditis, leading to regression of CHF. A high intensity endurance exercise program could possibly worsen myocarditis and CHF. Since very few studies exist that are providing conclusive information about this topic these hypotheses are very speculative and in need of experimental prove. Treating subgroups of mice with MCP–1 antibodies and/or CCR2 blockers would provide even more information.

In conclusion, cardiac-specific overexpression of MCP–1 induces myocarditis causing the homogenous development of CHF. The determination of exercise capacity has proven to be a precise marker for the limitation of cardiac function in CHF. We could demonstrate that, without the presence of other symptoms like visible congestion and resting dyspnea, moderate exercise intolerance was present at an age of 16 weeks, indicative of a compensated stage of CHF. The switch from the compensated to the decompensated CHF stage occurs between 24 and 28 weeks of age of MCP–1 transgenic mice. Visible congestion and resting dyspnea are additional symptoms, which become apparent during this period.

Exercise capacity was then severely impaired, resulting either in death of the mouse during the exercise test or inability to run at the initial level of the test. Massive cardiac and atrial hypertrophy in combination with severe ascites and pulmonary congestion illustrate the development of decompensated CHF.

6.4.2. Time Course after Bilateral Femoral Artery Occlusion

Measurements of collateral dependent foot blood flow by LDI give rise to relative numbers, which indicate that foot blood flow of the occluded leg is at the level of a certain percentage of foot blood flow of the normal leg. LDI measurements revealed that Balb/C mice increased collateral dependent foot blood flow only to 51% during four weeks after femoral artery occlusion. Collateral dependent foot blood flow of sv129 mice increased significantly during the same period when compared to Balb/C mice. After four weeks foot blood flow of the occluded leg increased to 92% of the normal leg. C57Bl/6 mice displayed an even faster and stronger recovery in collateral dependent foot blood flow. These mice had a foot perfusion of 97% after two weeks.

We hypothesized that these remarkable differences in the recovery of foot blood flow would lead to similar differences in the recovery of exercise capacity. In order to establish the recovery of exercise capacity as a marker of the increase in collateral dependent foot blood flow, we had to make sure that femoral artery occlusion severely influences exercise capacity. Therefore, we bilaterally occluded the femoral artery of six mice of each strain. Results obtained seven days after occlusion indicate that a severe limitation of exercise capacity was exerted by bilateral femoral artery occlusion.

However, to compare the relative blood flow data with the results from the exercise tests, we had to express these data in relative units as well. For this purpose, we determined the exercise capacity of six control mice of each strain without femoral artery occlusion and set this number to 100%. Exercise capacity of each time point was then expressed as the percentage of exercise capacity of control mice without femoral artery occlusion.

While Balb/C mice with bilateral femoral artery occlusion were able to increase their exercise capacity during four weeks only to 48%, sv129 and C57/Bl6 mice showed a recovery of 69% and 65%, respectively. Similar to the faster increase in foot blood flow, C57Bl/6 mice increased exercise capacity to 59% after two weeks. However, the recovery of exercise capacity did not even reach 70% in any of the

strains. Even though sv129 and C57Bl/6 mice recovered foot blood flow to approximately 100%, the recovery of exercise capacity was markedly less.

Therefore, the results of the present study indicate that the recovery of collateral dependent resting foot blood flow is only partially reflected by the recovery of exercise capacity. Mice with the greatest recovery in blood flow (sv129 and C57Bl/6) demonstrate the greatest improvement in exercise capacity. However, their recovery in exercise capacity levels off at 68% to 65%, respectively, even though they entirely recover foot blood flow. Accordingly, mice with the smallest recovery in foot blood flow (Balb/C) show the smallest recovery in exercise capacity.

These observations give rise to several possible explanations. One explanation involves the significance of the resting foot blood flow measurements by LDI for skeletal muscle blood flow during exercise. Even though we observed in C57Bl/6 a total recovery of collateral dependent foot blood flow, this does not necessarily mean that skeletal muscle blood flow during exercise had increased to the same extend. Moreover, it is possible that skeletal muscle blood flow during exercise remained decreased when compared to control animals without femoral artery occlusion.

A study in rats performed by Yang et al. [177] points into this direction (see also chapter 6.3.). In their study not even rats with femoral artery occlusion trained for up to eight weeks were able to recover skeletal muscle blood flow to a level similar to control rats without occlusion of the femoral artery. The difference became especially apparent when the rats were running at high treadmill speeds. Trained rats with occlusion of the femoral artery showed only approximately 50% skeletal muscle blood flow during high-intensity exercise when compared to control rats. The difference was less during low-intensity exercise. Thus, it is possible that mice are capable to increase resting foot perfusion to 100% while skeletal muscle blood flow during high-intensity exercise is significantly decreased.

Therefore, future studies considering the relationship between the recovery of blood flow and the recovery of exercise capacity are in need of the determination of skeletal muscle blood flow during exercise. However, adaptation of the methods currently used for evaluation of blood flow will take some time and certain technical difficulties will have to be overcome. It is, nevertheless, worthy to do so because this technique can be used for other studies (see also chapter 6.3.).

Skeletal muscle blood flow measurements during exercise could provide information about a possible correlation of exercise capacity with skeletal muscle blood flow. It would be interesting to see if skeletal muscle blood flow during exercise is in better agreement with exercise capacity than resting foot blood flow determined by LDI. An endurance exercise program is able to increase skeletal muscle blood flow during exercise [177] and increases exercise capacity as well. If a correlation could be established, the real importance of skeletal muscle blood flow for exercise capacity could be uncovered.

One of the first observations of this study was the difference in the initial exercise capacity between the three strains expressed in absolute numbers (work in Joule). Normal Balb/C mice display the highest absolute exercise capacity followed by sv129 mice. C57Bl/6 mice displayed an absolute exercise capacity that was only slightly less than that of sv129 mice. This strain dependent difference of absolute exercise capacity could not be explained by any of the above mentioned observations. These findings provide material for future studies regarding anatomical and metabolic changes of mice of different strains leading to increased absolute exercise capacity.

Similarly, the reason for the difference in the recovery of collateral dependent blood flow is not presently known. Postmortem angiographies performed in our laboratory have recently shown that Balb/C mice display the typical corkscrew growth pattern of collateral arteries. Angiographies from sv129 and C57Bl/6 mice show a more diffuse picture of the developing collateral circulation. The angiographic findings suggest that Balb/C mice have a limited ability to grow and recruit more arteries for the blood supply of the hindlimb. It appears that they have to rely on the preexistent collateral arterioles and only the increase in diameter of these vessels provides the blood supply for the distal hindlimb. Thus, growth of a few collateral connections in size leads to angiographies with well visible collateral arteries in the typical corkscrew pattern. Especially C57Bl/6 mice that have the capacity to grow numerous arteriolar connections display rather diffuse angiographies without well visible collateral arteries in the typical corkscrew pattern.

These assumptions are in accordance with the recent angiographic findings of our study investigating the effect of general overexpression FGF–2 and endurance exercise (see also chapter 6.3.). Trained FGF–2 transgenic mice demonstrated the best gastrocnemius and foot blood flow and displayed diffuse angiographies. Sedentary nontransgenic mice with the worst perfusion had well visible collateral arteries in the typical corkscrew growth pattern.

However, the significance of these observations remains unclear. Therefore, histological analysis of the hindlimb tissue has to reveal the precise differences in collateral growth pattern. Differential gene expression arrays could indicate key genes responsible for the different recovery in collateral dependent blood flow.

In conclusion, our study provides evidence that different strains of mice with femoral artery occlusion display a different recovery of collateral dependent foot blood flow under resting conditions. This observation is reflected by a similar pattern of the recovery of exercise capacity, which does not reach the extent of the recovery in foot blood flow. C57Bl/6 mice demonstrate the best recovery of foot blood flow and exercise capacity. Compared to the other strains they increase foot blood flow and exercise capacity very rapidly. However, while resting foot blood flow increases to 100%, exercise capacity remains below 70% of control mice without occlusion of the femoral artery. Foot blood flow of sv129 mice was slightly less after four weeks when compared to C57Bl/6 mice. Exercise capacity was very similar after four weeks. Again, resting foot blood flow recovered to almost 100% but exercise capacity remained below 70%. Finally, Balb/C mice showed the worst recovery in foot blood flow (51%) as well as in exercise capacity (48%).

7. References

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