Molecular and cellular characterization of cardiac overload-induced hypertrophy and failure

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CHAPTER 1

General Introduction and Outline of the Thesis

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Part of this introduction is submitted for publication

General Introduction and Outline of Thesis

I. Normal Function

The heart contains two fluid pumps: the right heart pumps blood through the lungs and the left heart pumps blood through the rest of the body. Upon exercise, the heart can adapt its output through a unique property, being the length-dependent force generation. One pump cycle has four phases: the filling phase that has started with opening of the atrioventricular valve while the output valve is closed, the isovolumetric contraction phase occurring with both valves closed, the ejection phase during which the output valve is open whereas the atrioventricular valve is still closed, and the isovolumetric relaxation phase occurring with both valves closed (see Figure 1). During filling of the chamber the enlargement of the chamber by the incoming blood mass occurs at low filling pressures (≈5 mmHg), which demonstrates the compliance of the chamber during diastole. During systole the chamber shows a progressive stiffness, necessary to expel a mass of blood from the chamber in an artery with quite high blood pressure.

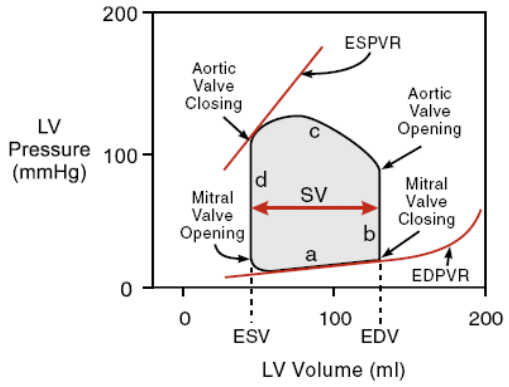

Figure 1. The pressure-volume loop describing one contractile cycle of the left ventricle. The four phases distinguished by the pressure-volume loop are the filling phase (a), isovolumetric contraction phase (b), the ejection phase (c), and the isovolumetric relaxation phase (d). ESV and EDV represent left ventricular end-systolic and end-diastolic volumes, respectively. SV represents stroke volume. ESPVR (the end-systolic pressure-volume relationship) represents the line that connects all end-systolic points of loops generated during changes of preload.

Thus, myocardial tissue should have compliant passive properties, given by relaxed cardiomyocytes and the extracellular matrix (ECM), whereas during systole the myocardial tissue should be stiff which is produced particularly by

contracted cardiomyocytes. The myocardial tissue consists of cardiomyocytes (≈75 vol%), interstitium (≈15 vol%), and capillaries, venules and arterioles (≈10 vol%).
For proper cardiomyocyte function, calcium homeostasis is of utmost importance. Ca^{2+} ions are released from the SR upon Ca^{2+} influx through the sarcolemmal L-type Ca^{2+} channels associated with phase 2 of the action potential. The Ca^{2+} release channels of the SR are blocked by ryanodine and are called the ryanodine receptors (RyR). Ca^{2+} release from the SR causes a rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) leading to formation of cross-bridges between myosin and actin, and contraction. Subsequent uptake of Ca^{2+} by the SR via the Ca^{2+}-ATPase pumps (SERCA) restores low diastolic Ca^{2+} concentrations, leading to relaxation [1].

ECM Composition, Synthesis and Degradation

The ECM is a network composed of fibrillar collagens, basement membrane components and proteoglycans. The major part of the ECM is synthesized by interstitial fibroblasts that are –in number- the most abundant cells in the myocardium. The ECM provides a scaffold for cardiomyocytes, fibroblasts, endothelial cells, and the vasculature to align and build a network. The weave of ECM proteins behaves quite elastic at stretch, but becomes stiff if stretched fully. Collagens type I and type III are the predominant interstitial collagens in the myocardium that generate structural integrity for the adjoining cardiomyocytes, providing the means by which cardiomyocyte shortening is translated into overall ventricular pump function. The network of collagen fibers exists at three levels: endomysium, epimysium and perimysium (see Figure 2). Endomysium surrounds individual muscle fibers, while the epimysium network surrounds a group of muscle fbers. The perimysium consists of thick, spiral-shaped bundles of collagen that connect epimysial and endomysial networks.
Basement membrane components include laminin, entactin, fibronectin, collagen type IV and fibrillin. Fibronectin and laminin are important for cell adhesion and cell-cell interaction. Proteoglycans include chondroitin sulfate dermatan sulfate, and heparan sulfate. Syndecans are transmembrane proteoglycans, with a family of four members, syndecan-1 to -4. The main protein comstituents of ECM of vascular tissue are collagen and elastin, with elastin being the most abundant protein in large arteries that are continually subjected to pulsatile pressures.

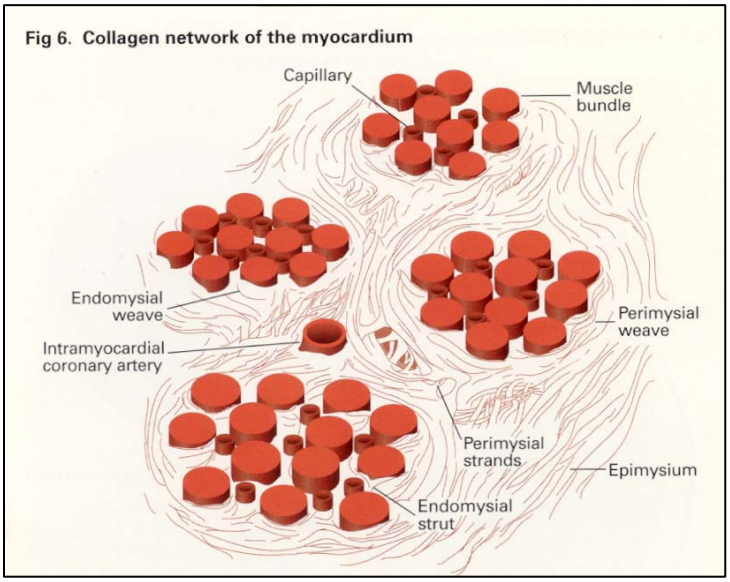

Figure 2. Collagen network of the myocardium (ref. 2).

Matrix Metalloproteinases

ECM integrity is maintained by a balance in the activity of matrix metalloproteinases (MMPs) (see Table), and their tissue inhibitors (TIMPs). The proenzymes of MMPs are secreted by several cell types, including cardiomyocytes. Proenzymes may be converted into active enzymes by other MMPs (e.g. by MMP14, or MT1-MMP) or by proteinases such as urokinase. Inflammatory cytokines, such as interleukin-1β, interleukin-6, and tumor necrosis factor-α, decrease collagen synthesis by cardiac fibroblasts, whereas anti-inflammatory cytokines, such as transforming growth factor-β, are potent stimulants of collagen synthesis. Cytokines also influence the synthesis and secretion of proMMPs, and MMPs are capable to process or activate cytokines from a latent form into an active form. This way, a complex feedback loop may occur whereby high MMP activity leads to increased cytokine bioactivity, further contributing to ECM remodeling.

MMPs are Ca^{2+} and Zn^{2+}-dependent proteases that are primarily synthesized as inactive zymogens (proMMPs), requiring activation by the removal of an amino-terminal propeptide domain either by autoproteolysis or processing by another MMP or serine protease. To date, 24 MMPs have been identified in vertebrates and classified in a number of ways; numerically, according to ECM substrate specificity, or based on shared functional domains (see Table 1). Beyond the ECM proteins, MMPs are now recognized to have non-ECM substrates including a number of growth factors and cytokines. Localization of a protease is an

important determinant in its actions. Membrane-type MMPs (MT-MMPs) are covalently linked to the cell membrane. Some of the secreted MMPs also localize to the cell surface by binding to an integrin [3], cell surface hyaluranon receptor CD44 [4], through interaction with cell surface associated heparin sulfate proteoglycans, collagen type IV, or the extracellular matrix metalloproteinase inducer (EMMPRIN) [5]. In the myocardium, MMPs are expressed by fibroblasts [6] and cardiomyocytes [7], and primarily function extracellularly [5]. In cardiomyocytes MMP2 is present where it colocalizes with troponin I and α-actinin along the Z-lines of the sarcomere [7,8].

Table. Matrix metalloproteinases in vertebrate tissues, including descriptive name and molecular weight of proenzyme and active enzyme.

MMP number	Descriptive name	Mol. weight (latent/active)	Substrate
MMP1	**collagenase**	**52/43 kDa**	**collagens (I, II, III, VII), basement membrane proteins**
MMP2	**gelatinase A**	**72/62 kDa**	**fragments of a number of collagens (I, III, IV, V)**
MMP3	**stromelysin**	**52/43 kDa**	**fibronectin, laminin, collagens (II, IV, IX)**
MMP7	**matrilysin**	**28/19 kDa**	
MMP8	**collagenase 2**	**75/55 kDa**	
MMP9	**gelatinaseB**	**92/82/65 kDa**	**fragments of a number of collagens (I, IV, V)**
MMP10	**stromelysin 2**	**52/44 kDa**	
MMP11	**stromelysin 3**	**51/46 kDa**	
MMP12	**macrophage elastase**	**52/20 kDa**	
MMP13	**collagenase 3**	**52/42 kDa**	**collagens (I, II, III)**
MMP14	**MT1-MMP**	**64/54 kDa**	**fibronectin, gelatin, laminin-I, fibrillar collagen**
MMP15	**MT2-MMP**	**71/61 kDa**	
MMP17	**MT4-MMP**	**62/51 kDa**	

Besides MMPs, certain metalloproteinases also contain a unique integrin-binding domain. These enzymes are called A Disintegrin And Metalloproteinase (ADAM).

To date, 34 ADAMs have been identified in a variety of species, with 19 in humans [9].These membrane-anchored enzymes bring about the shedding of numerous cell surface and matrix-bound proteins and are thus also called sheddases [10]. Among the cell surface molecules processed by ADAMs are growth factors, including heparin-binding epidermal growth factor (HB-EGF), and transforming growth factor-α (TGFα), and cytokines, such as TNFα, IL-1 and IL-6 [11]. These molecules are known to influence myocardial remodeling by introducing hypertrophy and/or apoptosis. Thus, the diversity of substrates now linked to metalloproteinase activity show that the role of MMPs and ADAMs in the myocardium extends beyond ECM degradation to their involvement in in cardiac structure, function and response to injury by regulating the release of ligands critical to cardiomyocyte hypertrophy and apoptosis.

Activity of MMPs is controlled by a series of endogenous inhibitors. TIMPs are specific MMP inhibitors in the tissue compartment and have a complex role. They reversibly inhibit activated MMPs through binding with MMPs in a 1:1 stoichiometry. There are four TIMPs in vertebrates. TIMP1 and TIMP3 are transcriptionally induced by growth factors and cytokines, while TIMP2 and TIMP4 are mostly constitutively expressed. TIMP1, TIMP2 and TIMP4 are present in soluble form, while TIMP3 binds to the ECM via heparin sulfate proteoglycans within the ECM [12]. TIMPs efficiently inhibit MMPs albeit with different specificity and affinity. In addition to inhibiting a broad spectrum of MMPs, TIMP-3 is also an effective inhibitor of ADAMs, as well as ADAMs with thrombospondin domain (e.g. ADAMTS-4 and ADAMTS-5) [13]. TIMP1 reportedly inhibits ADAM-10 and ADAMTS-1 [14].

Besides MMPs and ADAMs there is a protein called Extracellular MMP Inducer, abbreviated by EMMPRIN, also known under the name of basigin and CD147. It has been demonstrated that EMMPRIN is a cell surface glycoprotein that belongs to the immunoglobulin superfamily. It forms a complex with $\alpha_3\beta_1$-integrin [15]. It is highly expressed on the surface of tumor cells and stimulates adjacent fibroblasts or tumor cells to produce MMPs. EMMPRIN also stimulates expression of vascular endothelial growth factor (VEGF) and hyaluronan, which leads to angiogenesis and anchorage-independent growth/multidrug resistance, respectively [16].

Collagen synthesis in tissues is coupled to the release of aminoterminal propeptide of type I procollagen (**PINP**) and aminoterminal propeptide of type III procollagen (**PIIINP**) in blood. Collagen degradation in tissues is coupled to the release of carboxyterminal cross-linked telopeptide of type I collagen (**CITP** or **ICTP**) in blood (see Figure 3) [17].

The natriuretic peptides and NO, both of which function via the second messenger cGMP, demonstrate anti-fibrotic actions by inhibiting collagen synthesis and by stimulating MMP activity. On the other hand, norepinephrine, angiotensin II and endothelin-1 directly stimulate fibroblast proliferation and collagen synthesis, thereby increasing myocardial fibrosis [18,19]. In addition, aldosterone stimulates collagen synthesis in cardiac fibroblasts *in vitro* and in rat hearts *in vivo* [20,21].

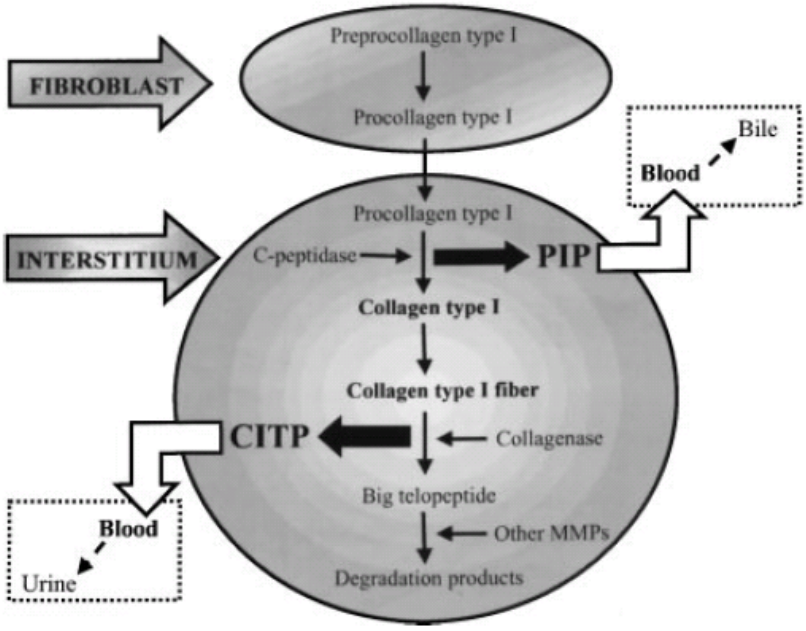

Figure 3. Schematic overview of collagen synthesis and degradation (ref. 17)

Excessive collagen synthesis leads to stiffness of the chambers and consequent impairment of diastolic properties, whereas overdigestion of the ECM results in myocyte-to-myocyte slippage and subsequent ventricular dilatation [22].
The connections of ECM and cardiomyocytes are located at the integrins.

Integrins and Integrin Signaling

Myocardial tissue comprises of fibers of serially coupled cardiomyocytes with parallel running capillaries embedded in a weave of extracellular matrix, mainly composed of collagen. The cardiomyocytes bind to the extracellular matrix by integrin receptors. Integrins are transmembranous heterodimeric receptors composed of an α- and a β-subunit. At the integrin receptors, the extracellular forces (via extracellular matrix), e.g. myocardial stretch, are transferred to the cell where the cellular cytoskeleton, including microfilaments, microtubules and intermediate filaments, maintains the shape and structure of the cell. The cytoplasmic terminus of integrin interacts with the cytoskeleton via a number of proteins that are part of the molecular machinery initiating the signaling response. These proteins include talin, vinculin, α-actinin, paxillin, filamin, zyxin, p130CAS, Src, focal adhesion kinase (FAK) and melusin. FAK is a cytoplasmic tyrosine kinase playing a major role in integrin signaling [23]. Clustering of integrins leads

to the recruitment of FAK to the cell-matrix adhesions and results in activation of FAK via autophosphorylation at Tyr-397. Even soluble integrin receptor ligands, the so called RGD peptides that contain the -Arg-Gly-Asp- sequence, could induce FAK phosphorylation in cardiomyocytes [24].
Several groups have demonstrated that soluble integrin receptor ligands cause a decrease of the L-type calcium currents or a decrease of $[Ca^{2+}]_i$ in arteriolar smooth muscle cells [25-27]. The $\alpha_5\beta_1$ integrin appears to regulate a Tyr-phosphorylation cascade involving Src and several focal adhesion proteins that control the function of the L-type Ca^{2+} channels [28].
Chan *et al.* reported that pretreatment with ryanodine (blocker of the ryanodine receptor (RyR) of the SR) completely eliminated the RGD-induced Ca^{2+} response, suggesting a role of integrin signaling and downstream effects on RyR, leading to Ca^{2+} release from the SR [29]. This result was corroborated by Van der Wees et al. who demonstrated that the RGD-induced Ca^{2+} release from SR is blocked by a NOS inhibitor, L-NMMA, while integrin stimulation by RGD was associated with elevation of intracellular NO, probably by NOS1, a NO-synthase isoform that is localized to the SR [30].
Ingber and colleagues proposed that upon extracellular forces integrins and cytoskeleton reorganise to form focal adhesions that transfer mechanical signals across the cell surface [31]. At these focal adhesion complexes several components of the cell's signal transduction systems are organised, including tyrosine kinases, inositol lipid kinases, ion channels, and certain growth factor receptors [32]. Signaling activities that have been shown to be modulated by mechanical distortion or fluid shear stress and to be mediated by integrins of focal adhesions in the cell include Src, focal adhesion kinase (FAK), extracellular receptor kinase-1 and -2 (ERK1/2), Shc, Grb2, protein kinase C (PKC), nuclear factor-κB (NF-κB), Akt, phosphatidyl-3-P kinase (PI3K), Ca^{2+} ions, cAMP, various stress-sensitive ion channels, actin polymerisation, and expression of genes encoding platelet derived growth factor (PDGF), endothelin-1 (ET1), and sterol regulatory element-binding protein-1 (reviewed in [33]). In skeletal muscle cyclic mechanical stretch induced the expression of β_{1D}-integrin, which, in turn, stimulated NO production and activated the downstream signaling proteins of the integrin pathway, FAK and RhoA. Activated FAK was assessed by FAK phosphorylation at Tyr397 and activated RhoA was assessed by activity assay. Stimulated NO production was considered to be due to NOS3 activation [34].

NO-Synthases and NO

NO-synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline + nitric oxide (NO). In the heart three NOS isoforms are present: NOS1, or neuronal NOS (nNOS), NOS2, or inducible NOS (iNOS), and NOS3, or endothelial NOS (eNOS). NOS1 and NOS3 are constitutively present enzymes and their enzyme activity is Ca^{2+}-dependent [35]. NOS2 is absent in the healthy heart, but its expression is induced by inflammation, mediated through cytokine-inducible transcription factors, such as IFN regulatory factor-1 and NF-κB to elements within the NOS2 promoter [36,37]. Massive quantities of NO generated by NOS2 induced by exogenously interleukin-1β, interferon-γ and lipopolysaccharide (LPA)

added to neonatal rat cardiomyocytes are shown to be toxic [38]. NOS2's enzyme activity is Ca^{2+}-independent.
As will be seen in the following paragraphs dealing about NOS isoforms and sub-cellular NOS compartmentalization, NO produced by a specific NOS isoform does not act as a freely diffusible messenger within the cardiomyocyte. In the tissue NO has two main effects: (***i***) NO stimulates the activity of guanylate cyclase, an enzyme that produces cGMP from GTP, and (***ii***) NO nitrosylates tyrosine residues in proteins and thiol-groups of cysteine in proteins. Upon nitrosylation proteins may change their properties, comparable to (but different from) the changes induced by phosphorylation, isoprenylation, geranyl-geranylation and palmitoylation [39-41]. Cellular proteins that may undergo S-nitrosylation are L-type Ca^{2+} channel [42-44], Kv1.5 channel [45], Ca^{2+}-activated ATPase of the sarcoplasmic reticulum (SR) [46], and the ryanodine receptor-2 of the SR [47,48]. This NO-induced post-translational modification of proteins serves as a major effector of NO bioactivity and an important mode of cellular signal transduction. Animals deficient in S-nitrosoglutathione reductase (GSNOR) show increased steady-state levels of circulating S-nitrosylated proteins at basal conditions and elevations of S-nitrosothiols in tissues following challenge by cytokines [49,50]. Thus, formation of S-nitrosothiols and their subsequent clearance are characteristics of NO-related signaling, and have analogies in phosphorylation of proteins by kinases and subsequent dephosphorylation by phosphatases. Thus, S-nitrosothiol turnover is considered to contribute to physiologic signaling. The systems affected most by GSNOR deficiency include the liver, immune system, and cardiovascular system.
NO exerts anti-apoptotic effects by S-nitrosylating and thereby inhibiting caspases 3 and 9, the kinase activities of both apoptosis signaling kinase-1 and c-Jun N-terminal kinase, and the transcriptional activity of jun [51-55].

NOS3

Under normal circumstances NO exerts several direct functions in the myocardium which are probably not related to the vasodilatory function of NO, such as acceleration of relaxation [56,57]. This effect is attributed to cGMP-dependent, protein kinase (PKG)-mediated phosphorylation of troponin I, leading to a reduction in myofilament Ca^{2+} sensitivity [58-60]. Apparently, these effects are exerted by stimulated NO release from vascular NOS3, as studies in hearts from $NOS3^{-/-}$ mice have not shown changes in myocyte relaxation [61], nor LV diastolic function [62], nor force-frequency response [63]. Myocardial NOS3 is mostly localized at the sarcolemmal and T-tubular caveolae, sites where caveolin-3 is also localized and where several signal transduction pathways have been shown to be modulated by NO [64]. Thus, sarcolemma-bound NOS3 inhibits the L-type Ca^{2+} channel and attenuates the β-adrenergic receptor-stimulated increase in myocardial contractility [62,65]. In mice with cardiomyocyte-specific NOS3 overexpression the line with highest level of transgenic NOS3 protein expression had increased heart weights. All lines displayed depressed LV peak systolic pressures, which was partially reversed by administration of L-NAME, suggesting

a net negative inotropic effect of NOS3-derived NO due to a blunted myofilament Ca^{2+} sensitivity [66].
NOS3 has been reported to co-purify with RyR [67] and to increase RyR open probability (Po) and the amplitude of the calcium transient under conditions of sustained myocardial stretch via a cGMP-independent mechanism [68].
Angiotensin-converting enzyme (ACE) inhibitors have been shown to enhance NOS3 expression and NO bioavailability (reviewed in [69]). Such a mechanism may contribute to the beneficial effects of ACE inhibitors in patients with heart failure. Moreover, NOS3 plays an important anti-atherogenic role: NOS3-derived NO inhibits (***i***) proliferation of rat aortic smooth muscle cells in a cGMP dependent way [70], (***ii***) monocyte adhesion to aortic endothelial cell monolayers [71], and (***iii***) collagen-induced platelet aggregation [72]. However, Garg & Hassid reported that NO from NO-donors like S-nitroso-N-acetylpenicillamine and isosorbide dinitrate, administered to BALB/c 3T3 fibroblasts, decreased DNA-synthesis and cell proliferation in these cells, without any effect on cGMP accumulation [73].

NOS2

Upregulation of NOS2 by interleukin-1β and interferon-γ increases apoptosis in neonatal rat cardiomyocytes by a process that is independent of guanylate cyclase activation and cGMP [74]. These authors also demonstrated that cytokine-induced apoptosis and peroxynitrite-induced apoptosis of cardiomyocytes are prohibited by treatment with a peroxynitrite scavenger. Generally spoken, NOS2-derived NO is considered to have detrimental effects on the myocardium. Mice with myocardial NOS2 overexpression suffered from cardiac fibrosis, cardiomyocyte death, cardiac hypertrophy, and cardiac dilatation. While a few NOS2-overexpressing mice developed overt heart failure, most animals died suddenly from atrioventricular block and asystole [75]. These data suggest that increased myocardial NOS2 activity is capable of initiating a process of cardiac remodeling that is characterized by ventricular hypertrophy, dilatation and sudden cardiac death.

NOS1

Myocardial NOS1 is normally localized at the sarcoplasmic reticulum (SR) membrane vesicles, where it influences the activities of calcium-handling genes [76,77]. The ryanodine receptor (RyR2) of the SR is also regulated by FKBP12 subunits, calmodulin, and protein kinases [78].
Myocardial NOS1 stimulates SR Ca^{2+} release and reuptake, facilitating Ca^{2+}-induced Ca^{2+} release and potentiation of the cardiac force-frequency response [63,79], probably by S-nitrosylation of calcium-handling proteins.
Accordingly, in $NOS1^{-/-}$ mice β-adrenergic receptor stimulation elicits a smaller LV inotropic response compared to control mice [65,80]. $NOS1^{-/-}$ mice had increased basal contraction, both in isolated LV cardiomyocytes as in their hearts in vivo. NOS1 disruption increased Ca^{2+} current and prolonged the slow time constant of inactivation of I_{Ca} significantly, leading to an increased Ca^{2+} influx and a greater

calcium load in SR in NOS1$^{-/-}$ cardiomyocytes [77]. Also the Ca^{2+} transient peak amplitude was greater in NOS1$^{-/-}$ cardiomyocytes than in cardiomyocytes from wild-type mice. The contractile response to β-adrenergic stimulation was greatly enhanced in NOS1$^{-/-}$ cardiomyocytes as well as in cardiomyocytes from wild-type mice treated with a specific NOS1 inhibitor, vinyl-L-*N*-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO) [81].

NOS1 inhibits xanthine oxidoreductase activity, both enzymes co-localizing in the SR. Thus, in NOS1$^{-/-}$ mice xanthine oxidoreductase-mediated production of oxyradicals (ROS) is increased, leading to depression of myocardial excitation-contraction coupling [82]. NOS1 deletion or inhibition leads to increased Ca^{2+} current through the L-type Ca^{2+} channels and reduction in SR SERCA2A activity, leading to increase of contraction and impairment of relaxation [57]. However, the positive force-frequency relationship in wild type mice is considerably attenuated in NOS1$^{-/-}$ mice, which suggests that NOS1-derived NO may enhance the force-frequency relationship [63]. This suggestion was rejected when the same authors found out that the force-frequency relationship in NOS1$^{-/-}$ mice normalized upon administration of allopurinol, an inhibitor of xanthine oxidoreductase [82]. Thus, Khan and coworkers concluded that a ROS-mediated reduction in myofilament Ca^{2+} sensitivity may be the mechanism underlying the reduced force-frequency relationship in NOS1$^{-/-}$ mice [82].

Isolated cardiomyocytes from mice with myocardial-specific overexpression of NOS1 demonstrated lower Ca^{2+} current density, lower amplitude of the Ca^{2+} transient, and lower amplitude of cell shortening, while their hearts in vivo revealed reduced contractility as judged from lower LV ejection fraction and lower dP/dt_{max} compared to nontransgenic littermates [83].

NO activates skeletal and cardiac ryanodine receptors, thereby regulating force in striated muscle [84]. NO-induced S-nitrosylation of ryanodine receptor-2 of the SR can increase the RyR open probability (P_o) through this mechanism [47,48]. There is evidence that S-nitrosylation of RyR2 and the L-type Ca^{2+} channel is preferentially mediated by NOS1-derived NO [85]. However, also NOS3 has been reported to co-purify with RyR [67] and to increase P_o of RyR as well as the amplitude of the calcium transient under conditions of sustained stretch via a cGMP-independent mechanism [68].

Like many signaling proteins, the activity of NOS1 can be regulated by phosphorylation. If phosphorylated by calcium calmodulin-dependent protein kinase II (CaCMKII), NOS1 activity diminished, but after phosphorylation by protein kinase C (PKC) NOS1 activity demonstrated a modest increase [86].

II. Hypertrophy

Upon continuous overload of the heart, either by pressure or by volume, the myocardial tissue undergoes adaptation-like reactions of which growth is the most prominent. This myocardial growth is mainly produced by growth of the existing cardiomyocytes (cell hypertrophy), whereas the non-myocytes of the myocardium, e.g. the fibroblasts, undergo hyperplasia to keep non-myocyte density more or less constant. Other reactions observed upon initiating myocardial hypertrophy is the expression of genes encoding proteins that were expressed in the fetal stage

only, such as atrial natriuretic factor in ventricular tissue and α-skeletal muscle-actin in ventricular tissue, whereas several other genes are downregulated (e.g. Ca^{2+}-activated ATPase of the sarcoplasmic reticulum [87]) or upregulated (e.g. Na^{+},Ca^{2+}-exchanger, β-myosin heavy chain). Although gene expression profiles in hypertrophied myocardium of rats subjected to pressure overload and to volume overload are similar for several genes, such as increased expression of B-type natriuretic peptide, lysyl oxidase-like protein-1 and metallothionein-1, other genes had overload-specific expression changes [88].
The hypertrophic ventricle suffers, due to a thicker wall, from impaired diastolic filling, leading to higher filling pressures.
A special type of cardiac hypertrophy is the hypertrophy usually observed in athletes of endurance sports, such as elite cyclists. These athletes have a "physiologic" hypertrophy that serves their exercise well, being large chambers that can fill with large volumes of blood and with preserved contractile properties. At rest, these athletes have a cardiac output that is produced by ≈40 beats per minute.
A cell model of mechanical loading is the *in vitro* mechanical stretch model of cultured NRCMs. Upon stretch of these cells intracellular Ca^{2+} concentration increases [89], thereby activating several Ca^{2+}-dependent processes, such as calcineurin activation [90]. This Ca^{2+}/calmodulin-activated phosphatase is responsible for dephosphorylation of NFAT3 leading to nuclear translocation and transcriptional activation of numerous hypertrophy genes [91].

ECM Composition, Synthesis and Degradation

The extent of ECM remodeling depends partly on a balance between pro-inflammatory and anti-inflammatory cytokines, which can be differentially activated depending on the type of myocardial insult or the stage of disease progression. Pressure overload-induced myocardial stress is associated with increased concentrations of inflammatory cytokines. Components of the ECM, such as fibronectin, laminin and collagens I and III, are upregulated during in vivo hypertrophy [92-94]. Also, several matricellular proteins in the ECM are upregulated during cardiac hypertrophy, such as osteopontin and tenascin [95,96]. The extent of ANP expression in rat cardiomyocyte cultures that were stimulated with an α_1-adrenoceptor agonist appeared to be highly dependent of the ECM components present in the coating of the culture dishes, such as fibronectin and laminin [97].
In hypertensive heart disease, fibrous tissue accumulation in the myocardium leads to myocardial dysfunction and heart failure. To assess efficacy of therapeutic strategies changes in myocardial collagen turnover should be monitored. To this purpose, the use of serological markers of collagen turnover, such as aminoterminal propeptide of type I procollagen (PINP), aminoterminal propeptide of type III procollagen (PIIINP) and carboxyterminal cross-linked telopeptide of type I collagen (CITP or ICTP), are extremely useful. The plasma level of PINP is significantly correlated to the collagen volume fraction in the myocardium of spontaneously hypertensive rats [98] and in the myocardium of hypertensive patients [99]. Treatment of spontaneously hypertensive rats with

quinapril [98] or losartan [100] led to (***i***) reduction of myocardial fibrosis, (***ii***) lower collagen volume fraction in the myocardium, (***iii***) lower plasma PINP concentrations, and (***iv***) a tendency to increased plasma ICTP concentrations. In hypertensive patients, treatment with losartan led to (***i***) lower collagen volume fraction in the myocardium, and (***ii***) lower plasma PINP concentrations [101].
As normally collagen synthesis is in equilibrium with collagen degradation [102], situations that disturb this equilibrium, like long-standing hypertension, are associated with abnormalities in either plasma PINP or PIIINP levels and/or abnormalities in plasma ICTP and/or MMP levels, and/or TIMP levels. In patients with hypertrophic cardiomyopathy myocardial collagen turnover is enhanced compared with controls, as reflected by higher levels of PINP, ICTP, MMP2, MMP9, and TIMP-1 in plasma of patients compared to plasma of controls [103]. The higher the plasma PIIINP level, the smaller was the LV end-diastolic diameter [103]. Quality of LV diastolic function, represented by the difference in duration between transmitral forward wave (A) and pulmonary venous retrograde (Ar) wave, was (***i***) directly related to plasma MMP1 and MMP2 levels, and (***ii***) inversely related to plasma PINP levels [103]. Although increased levels of MMP1 and MMP2 in plasma of patients with hypertrophic cardiomyopathy would compensate for increased plasma PINP and PIIINP levels, the increased plasma TIMP-1 level may explain why, in general, myocardial collagen turnover is enhanced in the patients associated with LV diastolic dysfunction.

Integrins and Integrin Signaling

In pressure-overload hypertrophy myocardial expression of integrins was increased markedly. The most abundantly expressed integrin in ventricular tissue, integrin β_{1D}, was upregulated together with integrin α1, α5, and α7. ANP mRNA was increased 6-fold, and myocardial concentrations of phosphorylated FAK, ERK1 and ERK2 were elevated [104]. In rat cardiomyocytes in vitro α_1-adrenergic stimulation resulted in cardiomyocyte hypertrophy, increased protein levels of integrin β_{1D} by a factor of 3-4, and rapid and sustained phosphorylation of FAK [105]. Also β_3-integrin plays a role in cardiac hypertrophy. In pressure-overloaded of rat RV β_3-integrin, c-Src and FAK associate in a cytoskeleton-bound complex [106]. Stimulation of β_3-integrin by **Arg-Gly-Asp** containing peptide in isolated adult cardiomyocytes triggers (***i***) activation of c-Src accompanied by binding to p130CAS and phosphorylation of FAK on Tyr925 [107] and (***ii***) activation of p70S6 kinase [108]. FAK has an important role in hypertrophic growth of cardiomyocytes. Upon pressure-overload myocardial FAK tyrosine phosphorylation is increased, associated with activation of c-Src and a number of downstream adapter and signaling proteins such as p130CAS, GRBS, PI3K and ERK1/2 [24,105,109-113]. Also mechanical stretch activates FAK and ERK1/2 [110,114]. FAK signaling also plays a key role in cardiomyocyte hypertrophy induced by α_1-adrenoceptor stimulation [105,111], endothelin-1 [112], and angiotensin II [115], all three being well known hypertrophic stimuli acting via G protein coupled receptors. Phenylephrine-mediated ANP expression is attenuated by the dominant negative FAK mutant, FRNK [105] and by a dominant negative form of integrin β_{1D} [97]. By overexpression of β_1-integrin in rat

cardiomyocyte cultures that were stimulated with an α_1-adrenoceptor agonist, protein synthesis and ANP expression increased ≈2-fold [97].
In response to increased hemodynamic load, ligand binding of integrins in the cardiomyocytes leads to their clustering. To propagate integrin signaling, focal adhesion complexes enriched in adapter and signaling molecules are subsequently assembled, leading to activation of pathways that are known to be implicated in the hypertrophic response. Studies that have used myocardial hypertrophy-inducing stimuli, like stretch [110], endothelin-1 [112], and phenylephrine [111], implicate a prominent role for integrins, integrin-dependent FAK multicomponent signaling complex, and downstream Tyr-phosphorylation of signaling molecules. The responses to these stimuli appear to be dependent of the type of ECM protein and the type of integrin receptor. Cultured neonatal rat ventricular myocytes that were stretched on deformable silicone sheet coated with collagen caused activation of the prepro-B-type-natriuretic peptide (preproBNP) gene promotor that was dependent of the presence of the integrin subunits β_1, β_3 and $\alpha_v\beta_5$ [116]. Stretch of cardiomyocytes can cause hypertrophy and induction of gene expression [117-120], as well as phosphorylation of FAK [121] and activation of the Ras/ERK1/2 pathway [119,122]. In pressure-overloaded rat heart, Franchini and coworkers observed a time-dendent increase in (***i***) FAK phosphorylation at Tyr397, (***ii***) c-Src phosphorylation at Tyr416, (***iii***) co-immunoprecipitation of FAK with actin, and (***iv***) phosphorylation of ERK/2 and Akt [109].
Overexpression of integrin β_{1D} or FAK triggers hypertrophy in NRCMs [105].

NO-Synthases and NO

NOS3

NOS3 normally generates NO, which can have antihypertrophic influences. However, pressure load results in NOS3 uncoupling associated with reduced tetrahydrobiopterin levels, transforming NOS3 activity to favor ROS generation [123]. In cardiomyocytes isolated from pressure-overloaded hearts NOS3 has reduced activity and expression [124]. Inhibition of endogenous NO formation induces myocardial hypertrophy [125]. Upon inhibition of NOS3-derived NO formation, adult rat ventricular cardiomyocytes showed an increase in protein synthesis and cell size [126], which confirms an earlier report demonstrating that $NOS3^{-/-}$ mice have hypertension and left ventricular hypertrophy [127]. In $NOS3^{-/-}$ mice pressure overload induced more severe left ventricular hypertrophy, LV dysfunction and myocardial fibrosis than in wild-type mice [128]. But if NOS3 was restored in the hearts of $NOS3^{-/-}$ mice, pressure overload by aortic constriction caused less LV hypertrophy and dysfunction than observed in NOS3-deficient mice [129]. Contradictory reports about the effects of NOS3 and NOS3-deficiency on pressure-overload LV hypertrophy and dysfunction have been published [123,130]. The former authors stipulated that NOS3 expression can have deleterious effects due to its uncoupling during pressure-overload, leading to

oxidant stress [123]. The latter authors stated that ANP production, associated with pressure overload, may prevent hypertrophy in NOS3-deficient mice [130]. Cardiac-specific overexpression of NOS3 attenuates the LV hypertrophy induced by isoproterenol infusion [131] and the LV hypertrophy induced by coronary artery occlusion [132].

NOS1

In cardiomyocytes isolated from pressure-overloaded hearts NOS1 undergoes upregulation [133].

NOS2

Mice with aortic constriction have induction of myocardial NOS2 expression and LV hypertrophy. NOS2-deficient mice demonstrated –upon aortic constriction– much less hypertrophy, dilatation, fibrosis and dysfunction, than wild-type mice with aortic constriction [134].

III. Heart Failure

Compensated hypertrophy is often seen to undergo a transition to heart failure [135]. Thus, although neurohumoral stimuli are accumulating to stimulate the heart to higher achievements, the heart is not able to do so leading to increased filling pressures and venous congestion. The failing heart has undergone many changes, the most prominent being changes in the calcium handling proteins [136]. The changing geometry, architecture and properties of the chamber are generally indicated as (adverse) chamber remodeling, in severe cases associated with atrioventricular valve insufficiency, dyssynchronous contractions of the chamber's wall segments, and rhythm disturbances. An important mediator of ventricular remodeling is the neurohumoral activation of the heart by the sympathetic nervous system and the renin-angiotensin-aldosteron system (RAAS). Full recognition of their deleterious influences on the heart has led to the foundation of the cornerstones of heart failure therapy: β-blockers and ACE-inhibitors (or angiotensin receptor type 1-blockers).
In the myocardial tissue the ECM undergoes a series of changes due to (***i***) myocardial collagen accumulation, (***ii***) collagen fibril disruption, (***iii***) altered arrangement and reduced cross-linking between collagen fibers, and (***iv***) synthesis of proteins that are not present in the healthy myocardium. To the latter group of proteins belong tenascin-C [137], thrombospondin-2 [138], matrix Gla protein [139], and osteopontin [139]. The volume percentage of ECM in failing myocardial tissue is usually increased, which is often accompanied by increased serum levels of aminoterminal propeptide of type I procollagen (PINP) and aminoterminal propeptide of type III procollagen (PIIINP), two propeptides that are liberated from the tissue upon collagen type I and type III synthesis. At the same time serum levels of carboxyterminal cross-linked telopeptide of type I collagen (ICTP) are low, representing a low rate of collagen degradation [17]. These

changes in ECM composition are ascribed to the myocardial fibroblasts that are stimulated by high plasma levels of catecholamines, angiotensin-II and aldosteron. High levels of collagen degrading enzymes, such as collagenases, may lead to increases in LV dimensions and subsequent cardiomyocyte slippage that may contribute to progressive LV remodelling [22]. In an animal model of hypertensive heart disease, increased expression of collagenases coincided with the transition from hypertrophy to heart failure [140].

During cardiac overload, calcium homeostasis becomes disturbed leading to an increase of diastolic $[Ca^{2+}]_i$ and a decrease of systolic $[Ca^{2+}]_i$. One of the underlying mechanisms is a decreased activity of SERCA. Recently, also the changes in cardiac RyR (RyR2) function are becoming elucidated. RyR2 comprises a tetramer composed of 4 RyR2 monomers, each binding 1 molecule of FKBP12.6 (also known as calstabin-2) [141]. Other proteins binding to the RyR2/FKBP12.6 complex are protein kinase A (PKA), the protein phosphatases PP1 and PP2A, and the anchoring protein mAKAP. In failing hearts phosphorylation of RyR2 by PKA causes dissociation of FKBP12.6 from the channel resulting in altered channel function manifested as an increased open probability (P_o) of RyR2 [142]. Secondly, RyR2 may become S-nitrosylated by SR-associated NOS1, which may influence P_o [47]. In failing cardiomyocytes an increased diastolic $[Ca^{2+}]_i$ and an increased Na^+,Ca^{2+}-exchanger (NCX) may stimulate an electrogenic Na^+ influx leading to early and delayed afterdepolarizations, underlying life-theatening arrhythmias. In this respect it is worthwhile mentioning that fish oils, such as EPA and DHA, protect against lethal arrhythmias and reduce P_o of RyR [143]. Moreover, several mutations in the human RyR2 gene are associated with stress-induced ventricular tachycardia, leading to sudden cardiac death. In molecular and cellular studies these mutations exhibit gain-of-function Ca^{2+}-release properties following cell stimulation [144].

ECM Composition, Synthesis and Degradation

The oxidative stress in the failing heart, in combination with high plasma levels of catecholamines, angiotensin-II, aldosteron, and endothelin-1, induces the synthesis of several MMPs and TIMPs, via transcription factor binding elements in their promoters, like binding sites for NF-κB, AP-1, Ets, and GATA. Also pro-inflammatory cytokines may activate transcription factors, leading to induction of MMPs and TIMPs [145]. Besides transcriptional regulation, ROS can also post-transcriptionally activate MMPs, e.g. by activating proMMPs. In failing hearts of patients with ischemic cardiomyopathy (ICM) or idiopathic dilated cardiomyopathy (DCM) protein and mRNA expression of TIMP1 and TIMP3 were reduced, but TIMP2 expression was unchanged [146]. These alterations were associated with increases in myocardial MMP1, MMP2, MMP9, MMP13 and MMP14 protein concentrations [147-151]. Right ventricular biopsies from hearts of patients with DCM demonstrated an inverse correlation between myocardial MMP2 and TIMP2 levels and the LV ejection fraction [152]. In patients with progressive heart failure, myocardial MMP1 and TIMP1 concentrations were significantly elevated compared to donor hearts [153]. In myocardium of patients with hypertensive

heart disease TIMP1 levels correlated with extent of fibrosis. This inhibitive effect on collagenase activity was associated with increased rates of collagen synthesis [154].

In myocardium of patients with heart failure several inflammatory cytokines, such as IL-1β, IL-6 and TNFα are elevated [155]. These increased proinflammatory cytokines can directly decrease collagen synthesis and procollagen mRNA expression in cardiac fibroblasts, while increasing the mRNAs of several MMPs as well as increasing the activities of several MMPs [156]. Activation of MMP activities by proinflammatory cytokines is amplified by the decreased expression of TIMPs by these cytokines [157]. The stimulating effect of proinflammatory cytokines on MMPs are attenuated by NO [158].

In myocardial biopsies from 20 patients with dilated cardiomyopathy (DCM), 5 patients with hypertrophic obstructive cardiomyopathy (HOCM), and 5 nonfailing donor hearts (control) DCM was associated with increased concentrations of ADAM-10, -15 and -17, while HOCM was associated with increased concentrations of ADAM-12 and -17, as compared to their respective concentrations in control myocardium [159].

In LV myocardium of 19 patients with aortic stenosis (AS) who underwent aortic valve replacement EMMPRIN mRNA contents were significantly higher than in myocardial tissue of 12 nonused donor hearts with normal LV function [160].

Plasma of patients with congestive heart failure (CHF) showed increased concentrations of tenascin-C and MMP9, that declined upon cardiac resynchronization therapy in patients who responded to this therapy by a decrease of LV end-systolic volume [137]. In addition, plasma of patients with CHF showed a 3-fold increase in MMP9/TIMP1 ratio and a 16-fold increase in MMP9/TIMP2 ratio, compared to plasma of a healthy reference population [161]. Another study reported higher circulating MMP2 in patients with severe CHF than in those with mild CHF, while in both groups circulating MMP2 levels were higher than in controls [162]. If MMP activities were inhibited with an MMP inhibitor, LV dilatation was attenuated in (***i***) the infarcted mouse heart [163], (***ii***) an animal model of cardiac volume overload due to an arteriovenous fistula [164] and (***iii***) a rat model of progressive heart failure, the spontaneously hypertensive heart failure rat [165].

In the heart's reaction to overload Janicki and coworkers recognized three phases. The **intitial phase** is characterized by an increased MMP activity associated with degradation of fibrillar collagen and development of progressive cardiomyocyte hypertrophy. In the **following phase**, the compensated phase, myocardial MMP activity and collagen concentration return to normal, while cardiomyocyte hypertrophy continues. The **final phase**, the decompensated phase, is attained once the compensatory mechanisms are exhausted and heart failure develops. This phase is characterized by elevated MMP activity, marked ventricular dilatation and prominent fibrosis [166]. During the decompensated phase, myocardial collagen content builds up despite elevated MMP activity due to massive collagen synthesis that is stimulated by multiple factors, including cytokines, angiotensin II, and high wall tension. The collagen synthesized may differ from that in the healthy heart with respect to type (e.g., type III instead of type I) and extent of collagen cross-linking.

These three phases were not observed in a porcine model of pacing-induced supraventricular tachycardia. In this model heart failure was induced that was progressive in the first 21 days after onset of pacing. During this period LV myocardial sections showed progressively lower collagen content and progressively higher activities of MMP1, MMP2 and MMP3, associated with progressively lower % fractional shortening of LV segments and progressively higher LV end-diastolic dimensions [167]. In patients with dilated cardiomyopathy the degree of diastolic dysfunction was associated with plasma PIIINP concentrations independently of LV volume and ejection fraction [168]. In these patients plasma PIIINP concentrations were correlated to (***i***) tissue collagen content [169], and (***ii***) poor outcome [170]. In patients with congestive heart failure resulting from LV systolic dysfunction event-free survival was predicted by plasma PIIINP level, LV ejection fraction and a restrictive mitral filling pattern [171].

Integrins and Integrin Signaling

As integrins trigger intracellular signaling pathways activating the cardiomyocyte hypertrophy program, mechanical signals and their integrin receptors likely contribute to transcriptional regulation of MMPs [172], expression of “fetal” genes, such as brain natriuretic peptide gene [173], and expression of genes encoding ECM proteins [174]. In a mouse model, cardiac-specific deletion of the integrin β_1 gene results in myocardial fibrosis and cardiac failure [175]. In myocardial biopsies taken from hearts of patients with ischemic cardiomyopathy there was a loss of integrin β_1D by 36%, a loss of FAK by 54%, a loss of phosphorylated FAK by 49%, and a loss of phosphorylated AKT by 44%, compared to myocardial tissue from individuals without cardiac disease [176]. Apparently, these results are not specific for failing myocardium, since myocardial biopsies taken from hearts of patients with idiopathic dilated cardiomyopathy were not altered with respect to integrin β_1D, FAK, FAK-P and AKT-P, as compared to myocardial tissue from individuals without cardiac disease [176]. In hearts of patients with ischemic cardiomyopathy the integrin β_1 mRNA concentration was unchanged compared to control hearts. The possibility that integrin β_1D has been shedded from the sarcolemma was tested and found to be unlikely as the observed loss of integrin β_1D in hearts with ischemic cardiomyopathy was not associated with an increase of a 55-kD integrin fragment [176].

NO-Synthases and NO

Dysregulation of NO and increased oxidative and nitrosative stress are implicated in the pathogenesis of heart failure [177,178]. Peroxynitrite is a reactive oxidant that is produced from the reaction of NO with superoxide anion and impairs cardiovascular function through multiple mechanisms, including activation of MMPs and nuclear enzyme poly(ADP-ribose) polymerase (PARP).
The induction of cytokines in the failing myocardium, such as IL6 and TNFα, induce the expression of NOS2 [37]. At the same time the production of reactive

oxygen species (ROS) is stimulated by NADPH oxidases, probably induced by increased angiotensinII levels [179].
The combination of abundance of ROS, impaired antioxidant defense mechanisms (superoxide dismutase, catalase and glutathione peroxidase) and reduced concentrations of antioxidants (vitamin E, ascorbic acid, glutathione) contribute to a state of oxidative stress, that together with increased NO formation, leads to formation of peroxynitrite [180,181]. By a process called NOS uncoupling, a monomeric form of NOS may become a source for myocardial ROS rather than NO [182-184]. The generation of peroxynitrite has been demonstrated in various forms of acute heart failure and chronic heart failure in both animals and humans [185, Table 2 of ref. 177] and in neonatal rat ventricular cardiomyocytes in culture peroxynitrite had detrimental effects [38]. Rats with chronic renal failure demonstrated marked elevations of blood pressure, plasma malondialdehyde, plasma nitrotyrosine, and tissue nitrotyrosine abundance, associated with depressed vascular tissue NO production and reduced immunodetectable NOS proteins in the vascular, renal and cardiac tissues [186].
Peroxynitrate has many effects [187], one of which being the induction of MMPs [188]. Overexpressing glutathione peroxidase [189] or administering tetrahydrobiopterin to decrease myocardial superoxide anion production [123] decreased myocardial MMP abundance.
In isolated aortic rings from rats with infarction-induced heart failure acetylcholine-induced vasodilatation was attenuated leading to a shift of the effect-dose curve to higher doses of acetylcholine [190].

NOS3

In cardiomyocytes isolated from canine hearts with hypertrophic cardiomyopathy NOS3 and caveolin have reduced expression [191]. Seddon and coworkers stated that "*to date it remains unclear whether constitutive myocardial NOS3 activity plays a role in regulating myocardial function in remodeled or failing hearts*" [57]. Although several groups reported a reduction of myocardial NOS3 expression and activity in failing myocardium of human hearts [192,193], other groups reported increased myocardial NOS3 expression and activity in failing myocardium of human hearts [194]. In biopsies taken from human failing hearts Fukuchi and coworkers demonstrated higher expression of NOS3 in cardiomyocytes of patients who were on β-blocker therapy than in cardiomyocytes of patients who were treated with β-adrenoceptor agonists [195]. Other groups found no change in LV NOS3 protein expression in infarcted mouse myocardium compared to sham-operated mouse myocardium [80], nor changes in myocardial NOS3 expression and activity in rats with volume overload-induced heart failure, compared to healthy rats [196].
Transgenic upregulation of myocardial NOS3 expression in mice with myocardial infarction was associated with a beneficial effect on LV remodeling [132]. NOS3 gene delivery protected mice with acute myocardial infarction against cardiac remodeling, myocardial fibrosis, apoptosis, and oxidative stress [197]. Apparently, NOS3 may counterbalance the deleterious effects of increased ROS in ischemia/reperfusion. Overexpression of human NOS3 in mice protected against myocardial infarction-induced congestive heart failure, including less pulmonary

edema and improved 1-month survival, compared to wild-type mice with myocardial infarction of equal size [198]. Correspondingly, NOS3 deletion had either detrimental effects (such as increased long-term mortality) or to have no significant impact on the development of LV failure in mice with myocardial infarction [199,200].
Oxidant stress from NOS3 uncoupling was reported to be responsible for pressure-overload LV remodeling and failure [123].

NOS2

Cardiomyocytes from patients and experimental animals with CHF have increased expression of NOS2 [192,195,196,201-203]. However, Stein and coworkers found no NOS2 mRNA in 28 of 30 failing human hearts, nor any NOS2 immunoreactivity in these hearts [194]. Only in failing hearts from patients with sepsis these investigators detected NOS2 protein expression [204]. NOS2 expressed in failing hearts is considered to lead to nitrosative stress, a pathophysiologic situation characterized by accumulation of S-nitrosylated proteins to hazardous levels. In addition, abundant NOS2-derived NO serves as a source of myocardial reactive oxygen species (ROS) that contribute to LV hypertrophy and dilatation [134]. Thus, myocardial NOS2-derived NO contributes to a cardiomyopathy phenotype that may exhibit a lethal brady-arrhythmia [75]. In myocardial biopsies from 22 patients with end-stage heart failure (8 patients with dilated cardiomyopathy and 14 patients with ischemic heart disease) Vejlstrup and coworkers detected NOS2 predominantly in vascular endothelium and smooth muscle cells, regardless of the etiology. Only in 4 of 22 patients with end-stage heart failure NOS2 was found in cardiomyocytes, associated with the sarcolemma [202]. In myocardial biopsies of 24 patients with end-stage heart failure NOS2 mRNA as well as NOS2 activity were increased. Myocardial NOS2 activity was inversely correlated with the inotropic response to isoproterenol [192]. This attenuation of inotropic effects to isoproterenol was associated with an accelerated relaxation in the failing hearts. The use of a general NOS inhibitor, L-NMMA, enhanced the inotropic effects of the failing hearts to β-adrenergic stimulation. In LV endomyocardial biopsies taken from 20 patients with dilated cardiomyopathy NOS2 mRNA and NOS3 mRNA concentrations correlated linearly with LV stroke volume and LV stroke work [205]. An intracoronary infusion of substance P, which releases NO from the coronary endothelium, increased LV stroke volume and LV stroke work, and shifted the LV end-diastolic pressure-volume relationship to the right, representing a concomitant increase in LV preload reserve [205]. Fukuchi and coworkers found that in biopsies of 28 failing human hearts increased NOS2 activity was mainly associated with infiltrated macrophages rather than with cardiomyocytes [195].
In mice with myocardial infarction myocardial NOS2 expression is increased associated with higher NO production and higher nitrotyrosine levels, leading to myocardial dysfunction and increased mortality. $NOS2^{-/-}$ mice with myocardial infarction had better contractility and lower mortality than wild-type mice with acute myocardial infarction [206]. Other studies corroborated the deleterious effects of NOS2-derived NO on infarcted myocardium. Sam and coworkers found that in NOS2-deficient mice late after myocardial infarction contractile dysfunction

was attenuated and apoptotic cell death was reduced [207]. However, Jones and coworkers reported that in NOS2-deficient mice severe congestive heart failure was not attenuated compared to wild-type mice with myocardial infarction [208]. Liu and coworkers found that myocardial infarction-induced increase in LV chamber dimension and the decrease in LV ejection fraction were less severe in $NOS2^{-/-}$ mice compared to wild-type mice [209]. Also myocardial concentrations of nitrotyrosine and 4-hydroxy-2-nonenal, markers for ROS, were lower in infarcted $NOS2^{-/-}$ mice compared to wild-type mice with myocardial infarction, indicating reduced oxidative stress by lack of NOS2-derived NO.
As mentioned before, induction of NOS2 by cytokines was found to cause cardiomyocyte apoptosis [74]. The increased expression of NOS2 in myocardium of animals and patients with heart failure [192,201-203] may be responsible for increased numbers of apoptotic cardiomyocytes observed in myocardium of animals and patients with heart failure [210,211].

NOS1

Myocardial NOS1 expression and activity have been reported to be increased following experimental myocardial infarction in rats [212,213], in mice [80], in human failing hearts [193], and in spontaneously hypertensive rats [191]. In failing LV myocardium NOS1 was found to be translocated from SR to the sarcolemma where NOS1 associates with caveolin-3 [193,212,213]. Irreversible activation of the RyR by oxidants leads to Ca^{2+} leak from SR, depressed calcium stores in the SR, and a heart failure phenotype [214].
Increased activity of NOS1 in failing myocardium does not necessarily translate in higher NO production rates, particularly if tetrahydrobiopterin levels and other cofactors are falling short. Then NOS1 uncoupling may occur, leading to ROS generation. In plasma of patients with congestive heart failure elevated concentrations of biomarkers reflecting oxidative stress have been observed [215]. Plasma concentrations of lipid peroxides increased with increasing NYHA classes of heart failure [216] and plasma concentrations of malondialdehyde-like material and plasma thiols correlated with LV ejection fraction negatively and positively, respectively [217].
Upon translocation to the sarcolemma, NOS1 exerts NOS3-like effects, such as inhibition of β-adrenergic receptor-stimulated increase in inotropy [212].
Inhibition of NOS1 enhanced the inotropic and lusitropic response to β-adrenergic stimulation in failing rat hearts but had no significant effect in sham-operated rats. Accordingly, myocardial NOS1 overexpression may contribute to the depressed β-adrenergic inotropic responsiveness observed in heart failure [213]. In $NOS1^{-/-}$ mice acute myocardial infarction caused more severe LV remodeling and impaired β-adrenergic reserve and increased mortality compared with wild-type mice with similar infarct size [80,218]. On the basis of these results Casadei suggested that NOS1-derived NO may delay the development of heart failure after myocardial infarction [219]. Since NOS1 suppresses xanthine oxidoreductase (XOD) activity under normal conditions, NOS1 deletion causes XOD activation and oxidative stress. In infarcted $NOS^{-/-}$ mice, oxidative stress is probably responsible for β-adrenergic hyporesponsiveness, depressed

myofilament responses to activator calcium, more intense hypertrophy of cardiomyocytes, adverse LV remodeling, and increased mortality [82,218,220].
In failing LV myocardium NOS1 was found to be translocated from SR to the sarcolemma where NOS1 associates with caveolin-3 [193,212, 213]. Following ischemia/reperfusion female mice exhibited increased NOS1 in association with caveolin-3 and increased S-nitrosylation of the L-type Ca^{2+} channel. Functionally, this led to decreased L-type Ca^{2+} current with reduced Ca^{2+} entry into the cell, which in turn protected the cell from calcium overload injury [221]. Due to translocation of NOS1 from SR to the sarcolemma of failing cardiomyocytes, suppression of XOD activity is relieved, which contributes to oxidative stress. S-nitrosylation of RyR2 is now considered to be beneficial for proper RyR2 function. In $NOS1^{-/-}$ mice cardiomyocytes were found to have higher diastolic Ca^{2+} concentrations compared to wild-type mice, suggesting diastolic Ca^{2+} leakage from SR. This Ca^{2+} leak from SR through RyR2 was associated with diminished S-nitrosylation and increased S-oxidation of RyR2 [222]. These authors concluded that the hyponitrosylation of RyR2 in NOS1-deficient mice is responsible for Ca^{2+} leakage from SR, leading to an arrhythmogenic phenotype.
In infarcted $NOS1^{-/-}$ mice worse remodeling and survival occurred despite an increased NOS3 expression [218]. The finding that tissue NO production was not elevated in these mice may be explained by NOS3 uncoupling contributing to nitroso-redox imbalance [223].
When both NOS1 and NOS3 genes are deleted, concentric hypertrophy is observed associated with interstitial fibrosis, impairment of LV diastolic properties, and high mortality [224].
Hearts of transgenic mice with cardiomyocyte-specific NOS1 overexpression subjected to pressure-overload by aortic constriction developed hypertrophy with thicker LV walls, less LV dilatation, and better preserved LV fractional shortening than hearts of wild-type mice with aortic constriction. Cardiomyocytes isolated from NOS1-overexpressing hearts with aortic constriction had higher amplitude of intracellular Ca^{2+} transients and higher SR Ca^{2+} load than cardiomyocytes isolated from hearts of wild-type mice with aortic constriction [225]. The negative effects of NOS1-knockout in conjunction with the positive effect of NOS1-overexpression on cardiomyocyte function before and during overload stronly suggest that cardiomyocyte NOS1 plays an important role in cell protection, particularly during conditions of cardiac volume or pressure overload.

IV. Experimental Models of Myocardial Hypertrophy and Heart Failure

Neonatal Rat Ventricular Cardiomyocytes (NRVCs) *in vitro*

The monolayer of NRVCs has proven to be a very useful cell preparation to study growth-promoting effects of conditions or substances on NRVCs *in vitro*. The capacity of pro-hypertrophic stimuli can be studied in terms of (***i***) increase in cell volume, (***ii***) expression of "fetal" genes, and (***iii***) sarcomeric organization. Simpson and colleagues have shown that α_1-adrenoceptor stimulation has potent

pro-hypertrophic effects on NRVCs mediated by protein kinase C and downstream signaling proteins [226-229]. Other pro-hypertrophic conditions or substances that have been tested in NRVCs *in vitro* are endothelin-1 [230], angiotensin II [231,232], myotrophin [233], and stretch [118,234-236].

Ventricular Pressure Overload *in vivo*

By inducing an increased afterload on left or right ventricle, the myocardium will undergo hypertrophy, characterized by (***i***) increased size of cardiomyocytes [237,238], (***ii***) increased number of interstitial fibroblasts to keep the fibroblast density more or less constant [238,239], and (***iii***) expression of "fetal" genes, such as the genes encoding ANP, skeletal α-actin and β-myosin heavy chain [240-242]. Even animals born with hypertension, the spontaneously hypertensive rats (SHR), have cardiac hypertrophy.

Due to multiple changes in gene expression a hypertrophic ventricle will undergo failure sooner or later [243], characterized by either ventricular dilatation, also referred to as ventricular remodeling, or myocardial fibrosis that opposes ventricular filling, also referred to as diastolic failure. Even SHRs will spontaneously develop heart failure at an age of 12-18 months [244].

Pressure overload of the RV is produced by either constriction of the pulmonary artery or induction of pulmonary artery hypertension (PAH).

Monocrotaline-Induced Pulmonary Hypertension

Mechanism and Pathology of Pulmonary Toxicity of Monocrotaline

Monocrotaline (MCT), a pyrrolizidine alkaloid derived from *Crotalaria spectabilis*, causes a pulmonary vascular syndrome in rats characterized by proliferative pulmonary vasculitis, pulmonary artery hypertension (PAH), and cor pulmonale. Current lines of evidence of the pathogenesis of MCT-induced pneumotoxicity indicate that MCT is activated to one or more reactive metabolites in the liver, particularly a MCT pyrrole called dehydromonocrotaline [245-247], and is then transported by red blood cells to the lung [248], where it initiates endothelial injury [249,250]. The endothelial injury does not appear to be acute cell death but rather a delayed functional alteration that leads to smooth muscle cell proliferation in the media of pulmonary arteriolar walls by unknown mechanisms. The role of inflammation in the progression of MCT-induced pulmonary vascular disease is uncertain. Both perivascular inflammation and platelet activation have been proposed as processes contributing to the response of the vascular media [247]. MCT and dehydroMCT are known to be toxic to a variety of domestic and laboratory animals and to humans. Major pathological effects induced by MCT poisoning include hepatic cirrhosis and megalocytosis, venocclusive disease, PAH, and RV hypertrophy. There is a positive correlation between progressive PAH, thickening of the medial wall of small pulmonary arteries and arterioles, and RV hypertrophy as a function of time [251].

Characterization of Right Ventricular Function during Monocrotaline-induced Pulmonary Hypertension in the Intact Rat

Besides RV hypertrophy, the primary response to MCT treatment is RV dilatation, i.e. increases of RV end-systolic and end-diastolic volumes and, consequently, a decrease of RV ejection fraction. The dose-dependent RV hypertrophy strongly correlates with MCT-induced pressure overload, but, despite this increased muscle mass, RV wall stress gradually increases, ultimately leading to RV decompensation [252]. Interestingly, RV end-systolic elastance and end-diastolic stiffness did not change significantly, even when corrected for myocardial muscle mass, suggesting that intrinsic myocardial function was not importantly altered. The unchanged RV diastolic stiffness was consistent with the absence of changes in fibrosis and the fact that filling pressures remained relatively normal. However, end-systolic and end-diastolic pressure-volume relationships showed a tendency to be shifted toward larger volumes, suggesting myocyte slippage as a potential mechanism for dilatation. In addition, early active relaxation, as reflected by τ, was severely depressed in the group of rats treated with 80 mg MCT/kg body weight, consistent with severe RV hypertrophy [252]. Using an orthogonal three-lead system, Henkens and coworkers recorded ECGs from rats with MCT-induced PAH at baseline, and 14 and 25 days after MCT administration [253]. Baseline ECGs of controls and MCT rats were similar, and ECGs of controls did not change over time. In MCT rats, ECG changes were already present on day 14 but more explicit on day 25: increased RV electromotive forces decreased mean QRS-vector magnitude and changed QRS-axis orientation. Important changes in action potential duration distribution and repolarization sequence were reflected by a decreased spatial ventricular gradient magnitude and increased QRS-T spatial angle. On day 25, RV hypertrophy was found, but not on day 14. They concluded that developing PAH was characterized by early ECG changes preceding RV hypertrophy, whereas severe PAH was marked by profound ECG changes associated with anatomical and functional changes in the RV. Three-dimensional ECG analysis appears to be very sensitive to early changes in RV afterload [253].

V. Purpose of the Study

We hypothesized that pressure overload is “felt” by the myocardium through stretch-like effects imposed on integrins, the receptor by which cardiomyocytes are attached to the ECM. In the cell model of NRVCs *in vitro*, we activate the integrins by administration of a Arg-Gly-Asp (RGD) containing pentapeptide to test whether integrin stimulation leads to NRVC hypertrophy. Any pro-hypertrophic effect of RGD-containing pentapeptide on NRVCs is compared with the well-known pro-hypertrophic effects of α_1-adrenoceptor stimulation with phenylephrine (**chapter 2**).

Ventricular failure is associated with a disturbed myocardial collagen turnover. In patients with heart failure, myocardial collagen turnover can be assessed by plasma concentrations of PINP, PIIINP, and ICTP that either represent measures of collagen synthesis (PINP, PIIINP) or collagen degradation (ICTP). We set out to investigate the effects of cardiac resynchronization therapy (CRT) on myocardial collagen turnover in patients with heart failure by comparing PINP, PIIINP and ICTP concentrations in plasma obtained at baseline and after 6 months of CRT (**chapter 3**).

As it is known for some time that MCT-induced PAH and RV failure were associated with activation of MMPs in RV myocardium, we first set out to investigate whether NO plays any role in PAH-induced RV hypertrophy and failure. To that purpose, two doses of MCT were used that produced RV hypertrophy only and RV hypertrophy and subsequent RV failure, respectively (**chapter 4**).

Which experimental treatments of PAH have been described? In **chapter 5** we review the novel approaches to treat pulmonary artery hypertension, particularly in experimental animals.

PAH is a life-threatening disease with an important pulmonary component that may provide a target to direct therapy. We set out to investigate whether MCT-induced PAH and subsequent RV failure can be treated with bone marrow-derived mesenchymal stem cells (MSCs) obtained from donor rats with PAH caused by injection of MCT 28 days earlier. At day 14 after MCT injection, recipient rats are treated with i.v. administration of MSCs from rats that had a MCT injection 28 days earlier (**chapters 6 and 7**).
In **chapter 6** the effects of stem cell therapy to rats with PAH on pulmonary pathology, incl. hypertension, are examined. In **chapter 7** the effects of stem cell therapy to rats with PAH on RV function and stucture are examined, including changes in myocardial extracellular matrix composition.

Cardiomyocytes isolated from RV myocardium of rats with and without PAH have been investigated electrophysiologically to explore PAH-related changes in excitability. In **chapter 8** we report on cardiomyocyte excitability properties dependent on Kv-channel expression, because these channels have been proposed to play an important role in PAH-related arrhythmias. We define the control excitability properties of RV cardiomyocytes from rats without PAH and make a provisional comparison with myocytes from PAH-rats in the discussion of this study.

By using *in vitro* cell preparations, rat hearts *in vivo* and *ex vivo*, and human plasma samples our goal is to characterize (***i***) the signaling pathways that are activated by pro-hypertrophic stimuli, (***ii***) changes in the ECM that are typical for heart failure, and (***iii***) the effects of cell therapy with MSCs on RV function, RV myocardial structure, and RV cardiomyocyte electrophysiology of rat hearts with MCT-induced PAH.

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