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The responses of cardiac cells to stress : focus on irradiation and mechanical stretch

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Citation

Wees, C. G. C. van der. (2006, January 24). *The responses of cardiac cells to stress : focus on irradiation and mechanical stretch*. Retrieved from <https://hdl.handle.net/1887/4268>

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**The responses of cardiac cells to stress:
focus on irradiation and mechanical
stretch**

Caroline Geertruida Christina van der Wees

Geboren te Amsterdam in 1973

The responses of cardiac cells to stress: focus on irradiation and mechanical stretch

proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D. D. Breimer,
hoogleraar in de faculteit der Wiskunde
en Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 24 januari 2006
klokke 14.15 uur

door

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Geboren te Amsterdam in 1973

Promotiecommissie

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Printed by: FEBO druk, Enschede

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ISBN-10: 90-9020277-3

ISBN-13: 978-90-9020277-8

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

The study described in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF-97-176).

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Preface and scope

Scope of the thesis

Excessive pressure or volume load of the heart, as seen in hypertensive and valvular heart disease, results in an adaptive growth response to compensate for the increased workload. The out coming cardiac remodelling leads to structural and functional changes, including enlargement of cardiac myocytes and an increase in fibroblasts and interstitial fibrosis (Swynghedauw, 1999). Initially these adaptations and functional alterations are beneficial; however, if they become insufficient to normalize wall stress, transition from compensated hypertrophy to heart failure may occur (Colucci, 1997). Increased stress, particularly mechanical stress, is the trigger off the conversion from compensated hypertrophy to heart failure, a process wherein various factors are considered to be involved.

The major objective of the studies presented in this thesis was to elucidate how cardiac cells cope with various stresses, mainly mechanical stretch and DNA damage. To examine the responses of cardiac cells to these stresses the studies described in this thesis made use of cultured cardiac rat myocytes and fibroblasts of neonatal origin. Primary cultures of cardiac cells, both myocytes and fibroblasts, were obtained from the hearts of 1-to-2 day-old Wistar rats. Cultures were prepared by dissociation of ventricular tissue, followed by pre-plating resulting in pure fibroblast and myocyte-enriched cultures.

A decrease in the number of myocytes, the cells responsible for the contractile function of the heart, may contribute to impaired contractility of the whole heart. Whether the loss of cardiac myocytes is the primary cause or the result of the progressive cardiovascular dysfunction remains unclear. Therefore in chapter two the direct effect of mechanical overload on the induction of programmed cell death, or apoptosis, in cardiac myocytes and fibroblasts was investigated.

Cardiac cells, both myocytes and fibroblasts, sense mechanical stretch and respond in a proper way to the stress. As integrins are candidate stretch-sensors of cardiac cells, the integrin function was examined by stimulating them with a synthetic pentapeptides containing the RGD sequence. Cellular responses, with regard to signal transduction pathways that couple the “sensing” message to “executive” measures, such as adaptation of gene expression, were measured and are described in chapter three.

It is known that genomes of all cells, including cardiac cells are the target of DNA damaging agents originating from multiple sources, both exogenous and endogenous. To cope with the deleterious consequences of DNA lesions, most cells

are equipped with efficient defence mechanisms. One such defence mechanism is removal of DNA damage by a DNA repair pathway that is called nucleotide excision repair (NER). Information about the expression of NER pathways in cardiac cells is scarce. Therefore the repair of DNA damage in cardiac myocytes and fibroblasts that had been submitted to UV-irradiation, a well known DNA damaging stimulus, was assessed (chapter four).

Increased stress, particularly mechanical stress, plays an important role in the conversion from compensated hypertrophy to heart failure. Therefore we applied micro array analysis in a study on mechanical stress imposed to cardiac myocytes and fibroblasts in co-culture and to cardiac fibroblasts in pure culture. The obtained information about mechanical stress induced changes in expression of a great number of genes is described in chapter six. To determine whether the changes in gene expression were reflecting a mechanical stress specific response or a common stress response the results were compared with those obtained after submitting the cardiac cells to two different DNA damaging agents, namely ionizing radiation and ultraviolet irradiation. The results suggest that mechanical stress, ionizing radiation and ultraviolet irradiation mainly induce stress-specific and cell-type specific gene expression profiles in neonatal rat cardiac myocyte-enriched cultures and cultures of neonatal rat heart fibroblasts. Functional groups that show significant percentages of differentially expressed genes suggest that certain cellular pathways are activated after one or more stresses (chapter seven).

1

Introduction

The heart

Structure and contractile function of the myocardium

The mammalian heart is a hollow muscle, made up of four pumping chambers, right and left atria and right and left ventricles that propel blood through the circulation, thereby delivering nutrients to and removing waste from the organs. Venous blood returning from the body is collected in the right atrium, passes via the tricuspid valve into the right ventricle and is then pumped into the lungs via the pulmonary valve. Returning blood, saturated with oxygen, enters the left atrium, passes into the left ventricle via the mitral valve and is ejected in the aorta via the aortic valve. The atrioventricular valves, situated between the atria and the ventricles, prevent the back flow of blood and are opened and closed by pressure changes between the heart chambers and the vessels (Katz, 2001). The blood supply to the heart itself arises from the left and right coronary arteries that originate from the aorta and run over the epicardial surface of the heart. The venous blood from the myocardial tissue is collected in veins that run parallel to the coronary arteries on the epicardial surface of the heart. Finally, the venous effluent of the heart enters the right atrium via the coronary sinus.

Cardiac pump function can be divided in four phases: (1) filling the pump chamber with blood, (2) isometric contraction to increase blood pressure in the chamber, (3) ejection of blood into the outflow artery, and (4) isometric relaxation leading to a fall of blood pressure in the chamber. The heart is able to enhance its contractile performance in response to an increase in volume by the so called Frank-Starling mechanism. The basis of this mechanism is length-dependent force development, that is to say increased filling of the ventricular chamber leads to increased ejection of blood. The cellular basis of the Frank-Starling mechanism involves the sarcomere length dependence of the Ca^{2+} vs. tension relation (Cazorla et al., 1999; Kentish et al., 1986).

Myocardial cells

In the myocardium several cell types are present, although the large cardiac myocytes make up most of the hearts mass. Approximately 70% of the cells in the heart are represented by relatively small non-myocytes, including vascular smooth muscle cells (VSMCs), vascular and endocardial endothelial cells (ECs) and

fibroblasts. Most of the cardiac non-myocyte cells belong to the fibroblast compartment that contributes to $\approx 50\%$ of the total cell number of the heart. The myocytes are responsible for the contractile function of the organ and constitute about 75% of the total volume of the myocardium. Myocytes are terminally differentiated cells in the adult myocardium that have lost their capacity to undergo cell division. In the rat heart for instance, myocyte division continues only until two weeks after birth (Beltrami et al., 2001), although recently in several cardiac diseases a small proportion of myocytes in the adult heart were shown to proliferate (Knaapen et al., 1997; Petrovic et al., 2000). The connective tissue, called the extracellular matrix (ECM) that contributes to the heart's tensile strength and stiffness, is produced largely by the fibroblasts. VSMCs form the muscle layer of the arteries and ECs form the inner lining of the endocardium, blood vessels and capillaries.

Structure and function of the ventricular myocyte

Myocytes contain large numbers of contractile proteins that are organized in myofibrils and are arranged in a regular array of thick and thin filaments. Muscle contraction results from sliding of actin filaments along myosin filaments by formation of cross-bridges, thereby using energy in the form of adenosine triphosphate (ATP). To this purpose a large fraction of the cell is occupied by mitochondria that oxidize substrates and consume oxygen, thereby regenerating ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) formed by cross-bridge cycling. Mitochondria and contractile filaments occupy almost 85% of the volume of the cardiac myocyte. Each myocyte is surrounded by a cell membrane, termed the sarcolemma. The sarcolemma not only lines the outer surface of the cardiac myocyte, it also penetrates into the intracellular space by a series of tubular invaginations. This extensive tubular network (T-tubules) increases the surface area of the cell and brings both intra- and extracellular domains in the closest contact possible (Ferrari and Opie, 1992). Because myosin and actin interact only in the presence of calcium, one of the major functions of the sarcolemma is to regulate the intracellular calcium concentration. For this reason the sarcolemma harbours ion channels, pumps and exchangers, such as Na^+/K^+ -pump, $\text{Na}^+/\text{Ca}^{2+}$ -exchanger and Ca^{2+} -channels. The sarcolemmal Ca^{2+} -, Na^+ - and K^+ -channels are involved in the generation of the action potential. Furthermore the sarcolemma contains specific receptors for hormones, neurotransmitters and growth factors (e.g. $\alpha 1$ - and β -adrenergic agonists, endothelin-1, angiotensin II,

transforming growth factor- β , insulin-like growth factor-I, and many more), GTP-binding proteins (Gs, Gi and Gq), several effector enzymes such as phospholipases (phospholipase A2 and phospholipase C), adenylyl cyclase, and translocated protein kinase C isoenzymes.

The Ca^{2+} movements within the cell are regulated by the sarcoplasmic reticulum (SR), a specialized form of endoplasmic reticulum that lacks ribosomes which makes it smooth in appearance. The SR plays a major role in controlling the free Ca^{2+} concentration in the cytosol.

Excitation-contraction coupling

Cardiac contraction and relaxation is controlled by an intracellular calcium cycle. During depolarization a small but variable quantity of Ca^{2+} ions enters the cell via L-type Ca^{2+} ion channels in the sarcolemma. Influx of Ca^{2+} ions induces a change in the molecular configuration of the calcium release channel of the SR, often indicated as the ryanodine receptor (RyR) since this receptor is sensitive to ryanodine. This calcium-induced change in molecular configuration “opens” the calcium release channel of the SR, and Ca^{2+} ions are released in the cytosol. The cytosolic Ca^{2+} concentration rises and contraction occurs. Contraction is followed by relaxation, which is the result of Ca^{2+} uptake by the SR through the activity of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), or the SR calcium pump (figure 1).

The Ca^{2+} ions taken up in the SR by SERCA are stored by the highly charged protein calsequestrin, a calcium-binding protein. Another storage protein, similar in structure and function, is calreticulin. Although both calreticulin and calsequestrin are present in the SR, these calcium-binding proteins display different patterns of distribution (Allen and Katz, 2000).

To prevent the myocardial cell from becoming overloaded with calcium, a small quantity of Ca^{2+} ions, equivalent to that entering the cell with each depolarization, is extruded. For this purpose the cardiac myocyte possesses two exit pathways, with the major route being the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism. When the cytosolic Ca^{2+} concentration exceeds a critical value and the voltage conditions are favourable, this mechanism extrudes Ca^{2+} ions. A back-up calcium extrusion system is the sarcolemmal calcium pump. This pump uses ATP to pump Ca^{2+} ions outwards and is switched on to function more actively when cytosolic Ca^{2+} concentration is high.

Mitochondria play a role in long-term buffering of calcium. Uptake of calcium into the mitochondria is mediated by a Ca^{2+} -uniporter driven by the mitochondrial membrane potential. Ca^{2+} ions are released from mitochondria by a mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

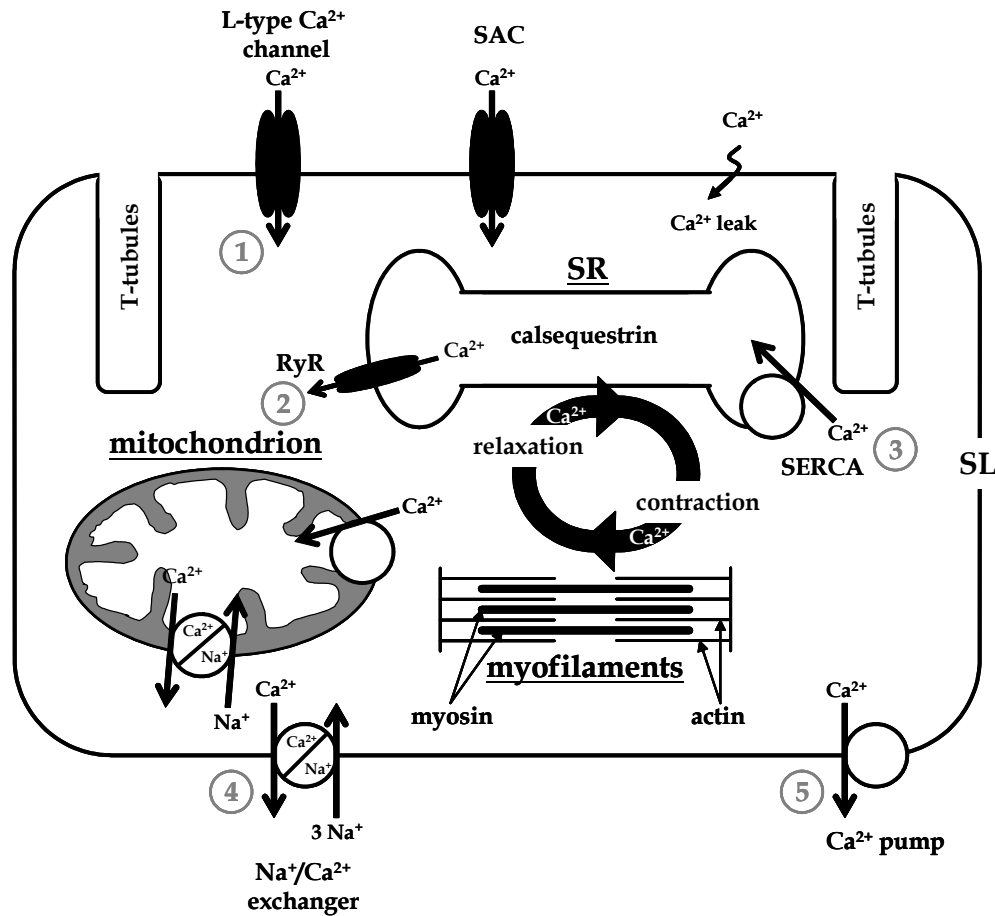


Figure 1. Schematic representation of calcium fluxes and cellular components involved in calcium homeostasis of cardiac myocytes. The main cellular organelles involved in calcium homeostasis are the sarcolemma (SL), sarcoplasmic reticulum (SR), the myofilaments and the mitochondria. During depolarization a small amount of Ca^{2+} ions enters the cell via the L-type calcium channel (1). These Ca^{2+} ions induce a change in the configuration of the Ryanodine Receptor (RyR) by which Ca^{2+} ions are released into the cytosol (2). Ca^{2+} ion uptake by the activity of the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) and storage by highly charged protein calsequestrin in the SR results in relaxation (3). To prevent the myocardial cell from becoming overloaded with calcium, a small quantity of Ca^{2+} ions is expelled by the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism (4) and the sarcolemmal calcium pump (5). Mitochondria play a role in long-term buffering of calcium. Uptake of calcium into the mitochondria is mediated by a Ca^{2+} -uniporter driven by the mitochondrial membrane potential. Ca^{2+} ions are released from mitochondria by a mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

Heart failure

Introduction to heart failure

Whenever the heart is chronically overloaded, myocardial tissue will try to adapt to these circumstances by myocardial growth, a phenomenon termed myocardial hypertrophy. This hypertrophic growth is termed compensatory when myocardial adaptations are sufficient to meet the demands of the body for blood. If the overload sustains and the adaptation and functional alterations become insufficient, transition from compensated hypertrophy to heart failure may occur (Bing, 1994). Heart failure is a syndrome with a spectrum of clinical manifestations that are primarily consequences of insufficient pump function of the heart. Heart failure also has the tendency to aggravate progressively. Factors responsible for progressive worsening of cardiac pump failure are maladaptive changes in myocardial cells, activation of renin-angiotensin system, progressive increase of systemic vascular resistance, increase of myocardial fibrosis, and apoptosis of cardiac myocytes. Patients with heart failure suffer from heart failure-associated arrhythmias, sepsis and worsening heart function, and have a high mortality rate. Basically there are three causes that can result in heart failure: pressure overload, volume overload and primary myocardial disease (cardiomyopathy). In all three cases the heart tries to compensate for the primary defects before the stage of heart failure develops.

In response to chronic pressure overload (due to systemic hypertension or aortic stenosis) peak left ventricular pressure increases to meet the increased afterload. The myocardium adapts to this overload by concentric hypertrophy, indicating that new sarcomeres (the contractile units) are synthesised in parallel with existing ones. The result is an increase of wall thickness without an increase of the ventricular diameter, thereby preventing wall stress to increase (figure 2).

Left ventricular volume overload may be caused by valvular regurgitation (incompetence) of either the mitral valve or the aortic valve. Due to valvular insufficiency there is back flow of blood (regurgitation), leading to an increase of stroke volume without an increase of cardiac output. The result of volume overload is eccentric hypertrophy, which is characterized by an enlargement of the cavity, without an increase in wall thickness. The myocytes demonstrate hypertrophy by synthesis of new sarcomeres in series with existing ones and adjacent myocytes may shift along the direction of stretch (figure 2).

In the third mechanism for cardiac failure (primary myocardial disease) myocardial force development is weakened at normal loading conditions of the left ventricle. In many cases the cause of the disease is genetic and associated with mutant forms of myofibrillar proteins. Dilated cardiomyopathy can also develop as a secondary phenomenon when the left ventricle is damaged by a large myocardial infarction or by severe ischemia of many segments. In fact, the non-ischemic part of the damaged left ventricle becomes overloaded and suffers from volume overload, leading to ventricular dilatation (remodelling).

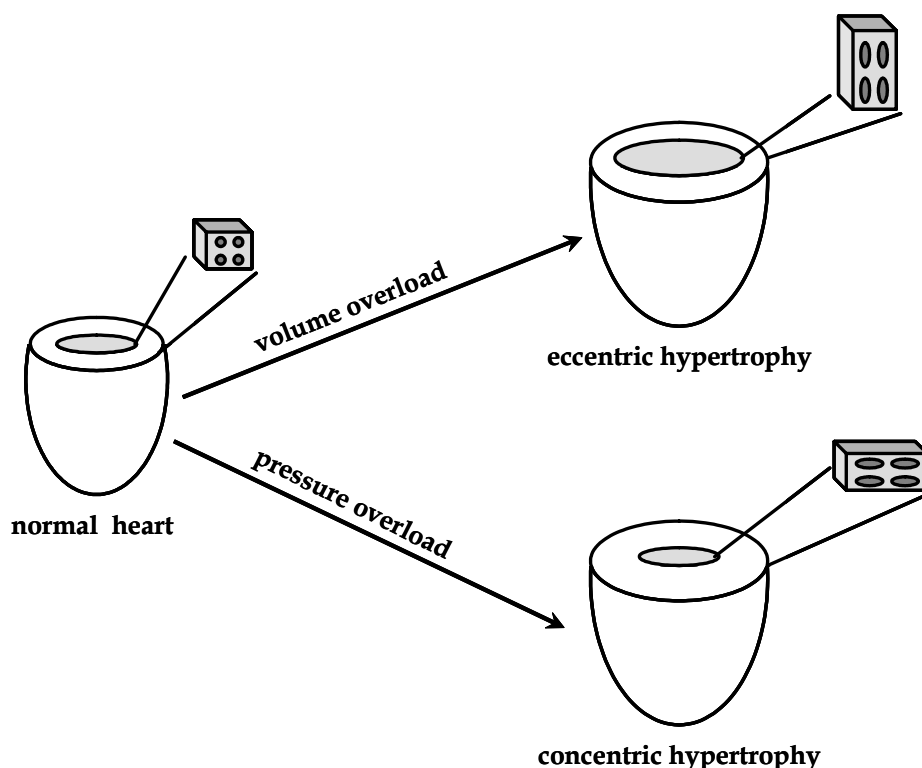


Figure 2. In volume overload the ventricle becomes hypertrophied by addition of sarcomeres in series. This will lead to enlargement of the ventricular cavity without thickening of the left ventricular wall and is called eccentric hypertrophy. The heart adapts to pressure overload by adding contractile elements in parallel, thereby increasing left ventricular wall thickness. This type of hypertrophy is referred to as concentric hypertrophy.

Moreover, several drugs and toxic substances may cause heart failure. Among these cardiotoxic drugs are members of the anthracyclins that are used to treat cancer. Anthracycline cardio toxicity is usually observed at a cumulative dose of

550 mg/m² body surface area, with a great variability in individual susceptibility and a dramatic increase with advancing age (Jensen et al., 2002). Whether this type of heart failure is reversible or not is not firmly established, but several small scale clinical studies have reported reversibility of left ventricular performance in patients with doxorubicin-induced heart failure after termination of therapy with this drug (Cohen et al., 1982; Saini et al., 1987).

Characteristics of myocardial and cardiac hypertrophy

Cardiac hypertrophy refers to supranormal growth of cardiac myocytes, although hyperplasia of fibroblasts is as important in the whole process, since these cells produce matrix constituents, such as collagen, that are responsible for increased stiffness and diminished compliance of the hypertrophied heart (Villarreal et al., 1998; Weber et al., 1992). During development of cardiac hypertrophy specific changes have been observed in cardiac myocytes: 1) rapid induction of proto-oncogenes and heat shock protein genes ("immediate-early" genes); 2) quantitative and qualitative changes in gene expression; and 3) increased rate of protein synthesis.

Proto-oncogenes

The first response to hemodynamic overload is the induction of proto-oncogenes (such as c-fos, c-jun, fra-1, Egr-1 and c-myc) and heat shock protein genes (such as hsp70), therefore called immediate-early (IE) genes (Isoyama and Nitta-Komatsubara, 2002; Pollack et al., 1994; van Wamel et al., 2000a). Proto-oncogenes are genes that can cause uncontrolled growth (oncogenesis). Their activation is tightly controlled in conditions of normal cellular proliferation. The products of proto-oncogenes are nuclear proteins that are able to bind to specific DNA sequences thereby inhibiting or activating the transcription of target genes (Simpson, 1988; Weinberg, 1985). Consequently, enhanced expression of these proto-oncogenes may be responsible for stimulating the overall rate of protein synthesis necessary for growth of the cells, but may also control alterations in expression of specific protein isoforms.

Changes in gene expression

As a later event in cardiac hypertrophy the expression of several genes is modulated, either quantitatively or qualitatively. Genes that were active in the embryonic stage but shut off soon after birth may become re-expressed in the hypertrophied heart. This reprogramming includes the expression of genes from, among others, a fetal repertoire, a skeletal muscle repertoire and an atrial repertoire. Associated with this genetic reprogramming are changes in a) contractile proteins, and b) in proteins involved in calcium homeostasis, generally leading to impairment of systolic and diastolic ventricular function. Ensuing calcium oscillations and triggered activity may cause electrical instability and sudden death (Janse, 2004).

a) Contractile proteins

The contractile machinery of the heart consists of proteins primarily concerned with contraction (actin and myosin) and proteins with a regulatory function (troponin and tropomyosin). In living muscle contraction will only take place in the presence of ATP and Ca^{2+} ions. Upon release of Ca^{2+} ions from the SR, intracellular Ca^{2+} ion concentration rises and contraction takes place. Relaxation is initiated by a rapid re-uptake of calcium ions by the calcium pump of the SR.

The transition of chemical energy into mechanical force involves a series of reactions that centre on the hydrolysis of ATP. The enzyme involved in these reactions forms an integral part of the myosin molecule and is known as myosin-ATPase. Myosin consists of two polypeptide chains termed the myosin heavy chains (MHC) and four polypeptide chains called myosin light chains (MLC). The site for the ATPase activity is present in the globular head of the MHC, which also carries the actin binding site. Since there are two types of MHC, the α -MHC and the β -MHC, three types of myosin can be found, myosin containing two α -MHC (V1), or two β -MHC (V3), or the heterodimer containing one α -MHC and one β -MHC (V2). The specific ATPase activity of these three MHCs decreases in the order V1, V2 and V3 (Alpert and Mulieri, 1986).

In rodents, left ventricular overload causes a shift in expression from α -MHC to β -MHC expression, which is considered to represent reintroduction of the fetal gene program (Huang et al., 2001; Iwanaga et al., 2000; Izumo et al., 1987; Spangenburg et al., 2002; Stephens and Swynghedauw, 1990; Swynghedauw, 1999). This shift in expression is associated with a lower myosin ATPase specific activity and

consequently a lower shortening velocity. This slowing improves efficiency of contraction (Alpert and Mulieri, 1982).

Also in the human hypertrophied heart a down-regulation of α -MHC and an up-regulation of β -MHC was observed (Lowes et al., 1997; Nakao et al., 1997; Reiser et al., 2001).

b) Calcium homeostasis

Several genes that encode membrane proteins are down-regulated in the hypertrophied hearts. The expression of SERCA and of its regulatory protein phospholamban is – after an initial increase – decreased after the onset of overload (Arai et al., 1993; Hashida et al., 1999; Matsui et al., 1995). As a result the intracellular calcium uptake by SR is slowed down, which may explain the reduced velocity of relaxation and the negative inotropy observed in hypertrophied hearts. As a compensatory response to the reduced calcium uptake of the SR, both mRNA and protein levels of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger are increased (Studer et al., 1997). In addition, the expression of the SR Ca^{2+} release channel, the RyR, is also decreased (Brillantes et al., 1992; Naudin et al., 1991). These abnormalities in calcium handling leads to a diminution (Gomez et al., 2001) and a prolongation (Beuckelmann et al., 1992) of the calcium transient (the cyclic variation in the concentration of intracellular Ca^{2+} ions per beat). The increased calcium concentration leads to activation of calcineurin, a calcium dependent phosphatase, that causes the nuclear translocation of the transcription factor NFAT and the initiation of a program of gene expression that leads to cardiac hypertrophy (Molkentin et al., 1998).

How do cardiac cells sense mechanical changes?

Integrins and integrin signaling

Individual cardiac myocytes are surrounded and interconnected by a pattern of weaves and struts that are formed by the extracellular matrix proteins (vitronectin, fibronectin and various collagens). This ECM provides a network of physical support that maintains myocardial structure and alignment throughout the cardiac cycle (Borg and Caulfield, 1981). A dynamic interaction between the cytoskeleton at the cell interior and the environmental components outside the cell is

accomplished by transmembrane receptors termed integrins. Integrins are composed of α and β subunit heterodimers that consist of a large extracellular domain, a transmembrane region, and a short cytoplasmic domain. The extracellular domain binds to proteins of the ECM or to counter-receptors on other cells, whereas the cytoplasmic domain forms links with cytoskeletal proteins and intracellular signalling molecules such as α -actinin and focal adhesion kinase (FAK) (Hynes, 1992; Laser et al., 2000; Ruwhof and Van der Laarse, 2000). Engagement of integrins with the ECM results in clustering and activation of the integrins on the cell surface, leading to the formation of so called focal adhesion sites.

Cardiac myocytes not only possess focal adhesion sites at their distal ends but also connections between the sarcolemma and sarcomeric Z-lines through cytoskeletal proteins. These sites are called costamers because of their “rib like” attachments and form the counterparts of the focal adhesion sites (Danowski et al., 1992; Pardo et al., 1983). This important structural site is linked to the ECM by integrins in the sarcolemma in the same manner as the focal adhesion site. Terracio et al. (Terracio et al., 1991) first demonstrated the presence of $\beta 1$ -integrins at the costamere surface of freshly isolated adult cardiac muscle cells. Costamere-like structures containing integrins are found in cultured neonatal and adult cardiac myocytes (Lu et al., 1992).

In addition to their structural role, focal adhesions and costamers are major sites for the localization of cell signalling molecules that are activated during cardiac myocyte hypertrophy (Franchini et al., 2000; Kuppaswamy et al., 1997; Laser et al., 2000).

Tubular invaginations of sarcolemmal membranes, termed T-tubuli, are located at the level of Z-lines (Terracio et al 1999). These inward extensions of extracellular space couple cellular excitation to contraction and may provide a venue for FAK-CAS-Paxillin activation by signalling molecules present in the extracellular space. The Z-line associated complexes are ideally positioned for regulation of sarcomeric contractile force, cytoskeletal organization and transduction of signals from ECM to the cytoplasmic and nuclear compartments, functions that are analogous to those largely subserved by focal adhesion sites in other cell types.

Tensegrity model of Ingber

In 1993 Ingber (Ingber, 1993) proposed a mechanical model of cell structure based on tension and integrity that could help to explain how cell shape, movement and

cytoskeletal mechanics are controlled. Besides, the model explained how cells sense and respond to mechanical forces. The model was termed tensegrity (tension and integrity) and refers to a tensed network that stabilizes itself mechanically by incorporating elements that resist compression. According to Ingber (Ingber, 2002), the microtubules in the cell act as compression bearing struts, the microfilaments are tension-bearers, and the intermediate filaments provide additional tensile connections in the structure that connect the cell surface to the nucleus. Those three networks work together with extracellular adhesions to prevent the cell from collapsing and maintain cell shape.

Cell surface integrin receptors, cytoskeletal filaments and nuclear scaffolds are mechanically coupled and stabilize nuclear structure (Maniotis et al., 1997). It is possible that through this mechanical coupling mechanical signals are transferred to the nucleus across the cell surface. Molecular connections between integrins, cytoskeletal filaments and nuclear scaffolds may thus provide a discrete path for mechanical signal transfer through cells as well as a mechanism for producing integrated changes in cell and nuclear structure in response to changes in ECM adhesivity or mechanics. Upon extracellular forces, integrins and cytoskeletal structures reorganise to form focal adhesions that transfer mechanical signals across the cell surface (Wang et al., 1993). Currently more than 50 proteins have been identified that are associated with the focal contacts and the related ECM adhesions (Zamir and Geiger, 2001). Most of these proteins contain multiple domains through which they can interact with different molecular partners, potentially forming a dense and heterogeneous protein network at the cytoplasmic faces of the adhesion site. Signalling activities that have been shown to be modulated in the cardiovascular system by mechanical distortion or fluid shear stress (and to be mediated by integrins of focal adhesions in the cell) include Src, FAK, ERK1/2, Shc, Grb2, PKC, NF- κ B, Akt, PI-3-kinase, calcium, cAMP, various stress-sensitive ion channels, actin polymerisation, and expression of genes encoding PDGF, endothelin-1 (ET-1), and sterol regulatory element-binding protein-1 (Ingber, 2002).

A prominent role for integrins, integrin-dependent FAK multicomponent signalling complex, and downstream Tyr-phosphorylation of signalling molecules in hypertrophy is implicated by studies that have used myocardial hypertrophy-inducing stimuli. The hypertrophic response of neonatal cardiac myocytes induced by phenylephrine (PE) is augmented by signals provided from the ECM (Taylor et al., 2000). This suggests that the signal transduction pathway leading to PE-induced hypertrophy is strongly influenced by and/or depend on integrin

mediated signal processes, including FAK. ET-1, another hypertrophy-inducing factor, induces assembly of focal adhesions and costamers and activates FAK (Eble et al., 2000). Moreover FAK is necessary for ET-1-induced hypertrophy, but other ET-1 generated signals are required to elicit the hypertrophic phenotype. Mechanical overload can be elicited by mechanical stretch and/or an increase in contractile activity. Strikingly, mechanical overload does induce the FAK multicomponent signalling complex, but an increase in contractile activity had no effect on the activity of FAK (Domingos et al., 2002).

The response to hypertrophic stimuli strongly depends on both the type of ECM protein and the type of integrin receptor. Expression of the brain natriuretic peptide (BNP) gene, which is often used as a hypertrophic marker, for example, demonstrated a dose-dependent increase after mechanical stretch when cells were cultured on fibronectin-coated deformable surfaces (Liang et al., 2000). Besides, this increase was dependent of the presence of the integrin subunits $\beta 1$, $\beta 3$ and $\alpha v \beta 5$ as was demonstrated by incubation with antibodies against the respective subunits. Even synthetic peptides containing the Arg-Gly-Asp (RGD) motif of fibronectin and vitronectin could induce FAK phosphorylation in cardiac myocytes (Laser et al., 2000). Thus by binding to components of the ECM and coupling to the cytoskeletal components in the cell, integrins play a central role in sensing stretching and compressive forces imposed to the cell. By initiating signalling transduction cascades, integrins are primary candidates to transduce forces (such as mechanical overload) into cellular reactions (such as hypertrophy).

Model system for mechanical stress

In vitro model of overload/ mechanical stress

A major factor considered to be responsible for the induction of cardiac hypertrophy is passive stretch. In the past, several models of hemodynamic overload that can be used for experimental investigations have been developed, such as: rats with pressure overload caused by aortic constriction, spontaneously hypertensive rats (SHR), paced papillary muscles, and isolated cardiac myocytes of both adult and neonatal origin exposed to mechanical stress.

The stretch device used to apply cyclic biaxial stretch was the Flexercell Strain Unit FX2000 (Flexercell Int. Corp., Hillsborough, NC, USA). This stretch device is based on the instrument described by Banes et al. (Banes et al., 1985). Flex plates, i.e. 6-

well plates with flexible bottoms made of silicone rubber, were put in a plate holder placed in a humidified incubator. The plate holder is connected to a vacuum pump, via a computer that controls pressure valves. When vacuum is applied to the bottoms of the flexible-bottomed stretch plates, the membranes are pulled down. As the cells are firmly attached to these membranes, stretch of these membranes results in stretch of the cells. Application of the vacuum results in maximum elongation of 20% to cells located at the periphery of the well, with strain declining towards the centre (Wung et al., 1996). The cells can also be stretched cyclically, i.e. each period of stretch is followed by a period of relaxation. The stretch device has several advantages: eight 6-well plates can be put in the stretch device at the same time, the stretch can be applied to cells in a humidified incubator, and the stretch regime can be controlled by a computer. A major disadvantage of the flexible membranes is that they are not optically clear, and therefore can hardly be used for microscopy and fluorometric investigations.

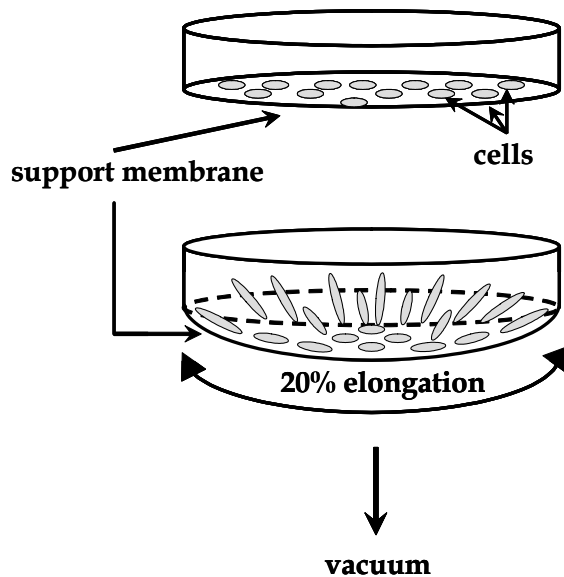


Figure 3. Schematic illustration of the *in vitro* stretch model. Cells are cultured onto flexible membranes. Upon applying vacuum below the membranes, they are pulled down and deformed. The cells, which are firmly attached to the membranes, are stretched by the deformation of the membranes. The vacuum pump is controlled by a computer, therefore cells can be subjected to several stretch regimes by changing the duration and the composition of a stretch cycle.

DNA damage and repair

DNA damage

DNA contains the blueprint for the proper development, functioning and reproduction of organisms ranging from bacteria, low eukaryotes to vertebrates

like man. The integrity of DNA is essential for the correct performance of these cellular processes. However the structure and integrity of the DNA molecules are under attack continuously by exogenous and endogenous (spontaneous) sources.

Attack by reactive oxygen species (ROS) is considered to be a major source of spontaneous DNA damage. There are various intracellular and extracellular sources of oxygen radicals, but the major intracellular source is probably the leakage associated with the reduction of oxygen to water during mitochondrial respiration. The products are singlet oxygen, superoxide radicals, hydrogen peroxide and hydroxyl radicals. Other spontaneous modifications can arise through alterations in the chemistry of bases (tautomeric shifts, deamination of bases). Modulation of oxidative stress has been suggested to play a role in multiple diseases such as cancer, cardiovascular disease, neurological degeneration and immune dysfunction. Furthermore, oxidative stress might play a role in ageing.

Physical and chemical environmental agents can also compromise the fidelity of DNA. The major physical agents are ionizing radiation (IR) and ultraviolet light (UV). IR can cause damage to all cellular components including DNA, thereby causing a variety of DNA lesions. Radiation damage to DNA is ascribed to direct and indirect effects (Ward 1998). Direct effects result from the direct interaction of the radiation energy with DNA. Indirect effects result from the interaction of DNA with the reactive species formed by radiation, predominantly hydroxyl radicals, superoxide and hydrogen peroxide. IR induces DNA damage including single- and double- stranded DNA breaks, apurinic sites and modified DNA bases.

Short wave UV (UV-C, 254nm) induces two major photolesions in DNA, i.e. the cyclobutane pyrimidine dimer (CPD) and the pyrimidine pyrimidone 6-4 photoproduct (6-4PP). The CPD, the most prominent photolesion, is formed by the covalent binding of two adjacent pyrimidines. The 6-4PP, formed in amounts that are approximately one third of the amount of CPD (Mitchell et al., 1990), is also formed at two adjacent pyrimidines by covalent linkage. This photoproduct introduces a more severe distortion in the double helical structure compared to the CPD (Taylor et al., 1988).

DNA repair

In virtually all organisms from bacteria to higher eukaryotes, several DNA repair pathways prevent the deleterious consequences of DNA damages. The biological significance of functional repair of DNA damage is apparent from the severe clinical features seen in individuals with repair-related disorders, such as

xeroderma-pigmentosum (XP), Cockayne syndrome (CS), trichothiodystrophy (TTD), Fanconi anemia (FA), ataxia telangiectasia (AT), Nijmegen breakage syndrome (NBS), and Bloom syndrome (BS). These patients have a mutation in a DNA damage response gene, leading to malfunctioning of a DNA repair pathway or to aberrant responses. The highly conserved DNA repair pathways are able to remove the vast majority of injuries from DNA. In mammals multiple DNA repair mechanisms exist, each with their own damage specificity and partly overlapping each other. Each repair system utilizes its own way of damage recognition, which, in most cases, depends on the effect the damage has on DNA, in terms of helix distortion, obstruction of DNA probing, or blockage of DNA replication or transcription (Hoeijmakers, 2001). Depending on the type of lesion, DNA damage can be removed by direct reversion, DNA strand break repair or its excision. Although reversal of damage in DNA is obviously the most direct mode of DNA repair, the types of damage that are repaired in this way are limited. The most general DNA repair mode is one in which damaged or inappropriate bases are excised and replaced by newly synthesized DNA. This type of repair is called excision repair and can be divided in base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR).

Base excision repair

The base excision repair machinery mainly removes bases with small chemical alterations and minor or no distortion of the double helix, such as lesions caused by spontaneous hydrolytic deaminations, reactive oxygen species, methylating or ethylating agents, and by DNA polymerase misincorporations (Friedberg et al., 1995; Lindahl, 1993). In contrast to NER, no universal recognition mechanism is employed within the BER pathway. Rather a set of distinct DNA glycosylases is utilized, each with specific affinity for a subset of related lesions, thereby functioning as a sensor for base damages. These glycosylases release the damaged base from the deoxyribose-phosphate chain, resulting in an abasic site. A single strand incision 5' to the abasic site opens the deoxyphosphate-backbone, and the base-less sugar is removed. This is followed by either one-nucleotide gap filling (short patch repair) or by removing a short stretch of DNA next to the damage, which is subsequently renewed (long patch repair) (Lindahl and Wood, 1999). Covalent integrity of the DNA is finally restored by a DNA ligase in both cases.

Mismatch repair

DNA mismatch repair (MMR) safeguards the integrity of the genome. It plays a prominent role in correction of mismatches generated during DNA replication which escape the DNA polymerase proofreading, mismatches that arise owing to spontaneous deamination of 5-methylcytosine, and mispairs that are formed during recombination (Buermeyer et al., 1999; Harfe and Jinks-Robertson, 2000; Schofield and Hsieh, 2003).

The fundamental understanding of MMR comes from extensive work performed on the methyl-directed MMR system in *E. coli* (Modrich and Lahue, 1996). The basic mechanism of MMR in all systems studied to date involves three steps: (I) recognition of the incorrect base, (II) excision of the misincorporated base and DNA surrounding the mismatch, and as a last step (III) DNA resynthesis and ligation of the nick.

In *E. coli* the mismatched base is recognized by the MutS protein, an ATPase that acts as a homodimer. In a reaction requiring ATP hydrolysis, MutL, together with the MutS-mismatched DNA complex, stimulate strand scission by MutH, to induce an initiating single stranded break on a hemi-methylated GATC site, that can be located at either site of the mismatch on the (newly replicated) unmethylated strand. MutL serves in several ways to couple mismatch recognition with downstream MMR events. In the first place a homodimer of MutL forms a complex with MutS and enhances ATP-hydrolysis dependent translocation (Allen et al., 1997). Next, MutL stimulate MutH endonuclease activity in an ATP-dependent manner (Hall and Matson, 1999). And third, MutL is required to load MutU (HelicaseII/UvrD) at the site of the MutH-induced nick, thereby facilitating DNA unwinding and subsequent exonucleolytic removal of the nascent strand. In the final step of the reaction, DNA polymerase III and SSB proteins perform repair synthesis to restore the original sequence.

Eukaryotes are known to contain an MMR system which has components that are highly related to key components of the pathway described for *E. coli*, although the mechanism of strand discrimination is different and not yet fully understood (Buermeyer et al., 1999; Harfe and Jinks-Robertson, 2000; Schofield and Hsieh, 2003). In eukaryotes 6 MutS homologues have been recognized (MSH1-6). MSH2, MSH3 and MSH6 form heterodimers MutS α (MSH2/MSH6) and MutS β (MSH2/MSH3) that are able to recognize mispairs in DNA (Buermeyer et al., 1999). MutS α preferentially binds most base/base mismatches and one base pair insertion/deletion loops (IDL), whereas MutS β preferentially binds 2-4 base pair

IDL (Kolodner and Marsischky, 1999). As in *E. coli*, after binding, a MutS homolog recruits a MutL homolog to the site of the damage. Multiple eukaryotic homologs of MutL have been identified in yeast and mammalian cells, MLH1-3 and PMS1, which function as a heterodimer. In contrast to the methyl-directed MMR system in *E. coli*, the identities of all required eukaryotic proteins have not yet been established. As in the *E. coli* system, after recognition of the mismatched base or loop, an excision reaction removes the mismatch or loop, and the DNA surrounding it. Based on genetic studies in yeast and the interaction reported between the yeast Msh2 and Exonuclease 1 (Exo 1) proteins, the human homolog to Exo 1, Hxx1/hExo1, may act as the excision nuclease in this process. Consistently, recent data have shown an interaction between the human Exo1-like protein and hMSH2. As a final step in the reaction, repair synthesis replaces the missing DNA.

A unique feature within this MMR pathway is the ability to distinguish between the correct nucleotide on the original template strand and the incorrect nucleotide within the newly synthesized strand. This capacity enables the process to remove the erroneous nucleotide and replace it by a correct one. The mechanism for strand discrimination *in vivo* for all organisms other than *E. coli* is unknown.

Nucleotide excision repair

Nucleotide excision repair (NER) is the most versatile repair pathway involved in the removal of a broad variety of structurally unrelated DNA lesions. Substrates for NER are helix-distorting lesions that interfere with proper base pairing, including UV-induced CPD and 6-4PP, bulky adducts induced by chemicals and several lesions produced by ROS (Friedberg, 1985; Moller and Wallin, 1998). NER comprises the following steps: (I) damage recognition, (II) open complex formation by melting of the DNA around the lesion and lesion demarcation, (III) dual incision of the damaged strand on both sides of the lesion, leaving a single stranded gap of 24-32 nucleotides, and (IV) gap filling by DNA synthesis and ligation (Figure 4). NER requires the concerted action of around 30 polypeptides, which act through the stepwise assembly of several subcomplexes at the site of the damage (Volker et al., 2001).

In the first step the lesion is recognized by the XPC-hHR23B complex. This DNA damage binding protein is essential for the recruitment of the NER components to the site of the DNA damage. In addition, the XPE protein is probably also involved in the damage recognition process (see below). Once bound to a DNA lesion, XPC-

hHR23B may induce conformational changes to the DNA helix that attract or facilitate the loading of other NER components, including the transcription factor TFIIH (Sugasawa et al., 1998). TFIIH is, apart from its role in basal transcription, a vital component of NER required for unwinding of the DNA helix at the vicinity of the lesion (Drapkin et al., 1994; Feaver et al., 1993; Schaeffer et al., 1993). The ATP-dependent helicase activity of the XPB-subunit of TFIIH is responsible for helix unwinding during RNAPII transcription initiation, whereas the helicase activities of both the XPB and the XPD-subunits are required for NER. Other basic NER factors that are required for the formation of a functional repair complex are the damage-binding factor XPA and the single-stranded DNA binding protein, replication protein A (RPA). The association of these two proteins to the pre-incision complex enables recruitment of the two structure-specific NER endonucleases XPG and ERCC1-XPF, which are responsible for the 3' and 5' incision in NER, respectively. Once the oligonucleotides that contain the lesion are removed, a DNA polymerase fills the gap and the nicks are sealed by DNA ligase.

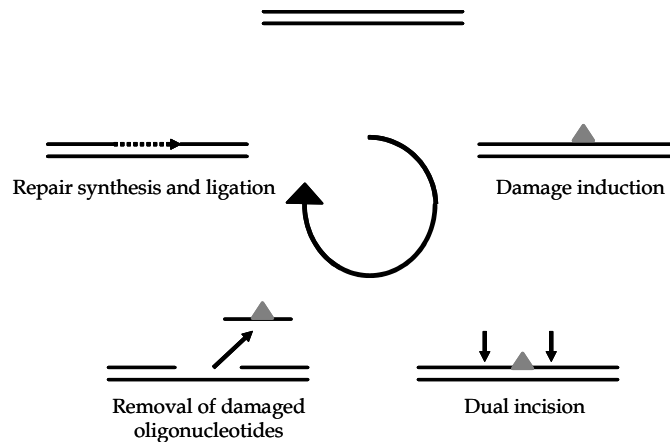


Figure 4. Schematic representation of nucleotide excision repair. After recognition of the damage, the damaged strand is incised at both sites of the damage. Subsequently the damaged oligonucleotides are removed and the resulting gap is filled up. Finally the newly synthesized DNA is ligated to the existing DNA.

Over 20 years ago Hanawalt and co-workers (Bohr et al., 1985; Mellon et al., 1986) discovered that the rate of repair of some DNA lesions in active genes is significantly different from that in non-transcribed regions, i.e. repair of DNA damage in active genes occurs much faster than in non-transcribed regions of the genome. Moreover, this faster repair is not simply due to the higher accessibility of DNA in transcribed regions, as it was subsequently shown that it is specifically the

transcribed strand that is preferentially repaired (Mellon et al., 1987). The non-transcribed strand of expressed genes is repaired with a rate that is similar to that of non-transcribed regions. The fact that only the transcribed strand of active genes is preferentially repaired forms the basis for the definition of transcription-coupled repair (TCR).

Within TCR the actual lesion sensing is performed by an elongating form of RNAPII, which is stalled at the lesion during transcription (Hanawalt, 1989; Hanawalt, 1990; Hanawalt, 1991; Venema et al., 1991), the XPC-hHR23B complex is not necessary in the damage recognition process. Although the damage recognition in TCR differs from that in Global Genome Repair (GGR) the actual removal of lesions is the same as in GGR. However, it is beyond the scope of this thesis to explain in detail the mechanisms by which the lesions are removed from DNA and the reason why the obstructing lesions in the transcribed strand of active genes are removed faster than non-obstructing lesions in the non-transcribed strand and elsewhere in the genome.

In summary two subpathways have been identified in NER, GGR, surveying the entire genome for helix-distorting injuries, and TCR, causing accelerated repair of lesions at which an elongating form of RNAPII is blocked during transcription.

Several specialized proteins have been proposed to play an important role during the initial step of damage recognition, including the UV-damaged DNA binding protein (UV-DDB) (Chu and Chang, 1988; Tang et al., 2000; Ho and Hanawalt, 1991). UV-DDB is a heterodimer of the p127 and p48 proteins (Keeney et al., 1993), products of the DDB1 and DDB2 genes, respectively. Recently, evidence has accumulated indicating that the XP group E phenotype is caused by mutations in the DDB2 gene, implicating that the DDB2 gene corresponds to the XPE gene, and p48 to the XPE protein (Itoh et al., 2000; Itoh and Linn, 2001; Rapic-Otrin et al., 2003). Noteworthy is the fact that binding activity and p48 expression are absent in wild type Chinese hamster cell lines (Rapic et al., 1998) and in several primary tissues from hamsters and mice (Tang et al., 2000). This given, together with the marked difference in CPD repair between human and rodent cells (rodent cells efficiently repair 6-4PP, but in marked contrast to humans, show almost no repair of CPD in the genome overall), has led to the notion that UV-DDB is essential for repair of CPD by GGR. Furthermore, the idea was strengthened by the observations that injection of purified human DDB protein into XP-E cells stimulated GGR to levels observed in normal human cells (Keeney et al., 1994), and transfection of hamster cells with human p48 gene enhanced GGR of CPD (Tang et al., 2000). Corresponding to this idea, in vitro binding studies have revealed that

the UV-DDB protein complex exhibits a high affinity for UV-induced DNA lesions and a moderate affinity for several other types of DNA lesions (Batty et al., 2000; Chu and Chang, 1988; Fujiwara et al., 1999; Payne and Chu, 1994). Moreover in vivo studies have shown that p48 and p127 relocalise rapidly to sites containing UV-damaged DNA immediately after irradiation, an observation consistent with a role for UV-DDB in UV damage recognition (Wakasugi et al., 2001).

Although DDB is essential for repair of CPD by GGR, the affinity of UV-DDB for 6-4PP is considerably higher than that for CPD (Batty et al., 2000), leading to the suggestion that UV-DDB might also contribute to GGR of 6-4PP, which greatly exceeds GGR of CPD. Moser et al (Moser et al., 2005) hypothesized that although 6-4PP can be repaired through a basal mechanism depending only on recognition by XPC-hHR23B, binding of UV-DDB to a 6-4PP will accelerate repair through enhanced recruitment of follow-up repair proteins. Since UV-DDB is degraded after binding to the 6-4PP, it cannot assist in subsequent repair of remaining 6-4PP and is therefore rate limiting. However, the impact of the rapid recruitment of UV-DDB to UV-damages on the efficiency of NER remains unclear (Fitch et al., 2003).

The efficiency of repair of DNA lesions by TCR and GGR in mammalian cells depends on a number of factors, including species, cell type and type of DNA lesion. Most notably, repair efficiency of UV-induced CPD appears to differ distinctly between rodent and human cells (van Zeeland et al., 1981). Most rodent cells are deficient in repair of UV-induced CPD in bulk DNA, and hence exhibit a defect in GGR of CPD. In contrast, TCR of CPD in these cells appears to be very efficient, resulting in selective repair of CPD in the transcribed strand of expressed genes (Vrieling et al., 1991). Human cells exhibit both GGR and TCR of CPD resulting in the preferential repair of CPD in the transcribed strand of expressed genes.

Nucleotide excision repair and terminal differentiation

Cells that do not divide and do not change their phenotype are known as terminally differentiated cells and are very common in multicellular eukaryotes. Since these cells do not replicate their genomic DNA, they could dispense themselves from the task of removing DNA damage from the non-essential bulk of their genome, as long as they are able to maintain the integrity of the genes that must be expressed. Nevertheless, terminally differentiated cells developed different ways to cope with DNA damage.

Neurons are probably the epitome of terminally differentiated cells: neurons do not proliferate and are not replaced by precursors. Terminal differentiation makes these cells a challenging experimental system and is the reason why many studies have been performed with tumor cell lines that can be differentiated into neuronlike or muscle like cells. Differentiation of human neuroteratoma NT2 cells into hNT neurons results in an almost complete loss of global repair of CPD (Nospikel and Hanawalt, 2000). Hardly any CPD removal occurred in a silent gene, whereas repair was very efficient in four active genes. No strand bias was observed, the non-transcribed strand being repaired much more efficiently than the rest of the genome. This peculiar repair phenomenon was termed differentiation associated repair (DAR). More recently, DAR was also observed in primary neurons (Nospikel and Hanawalt, 2002).

In the case of nerve cells it seems likely that cell turnover in the adult would be disadvantageous as a rule, since it would be difficult to re-establish the precise and complex patterns of nerve connections that were set up under widely differing circumstances during development. Another cell type that apparently has lost its ability to divide is the cardiac myocyte. Already in the early 1920s anatomical studies emphasized the difficulty of detecting mitotic figures in myocytes (Karsner, 1925). From then on evidence grew that muscle cell proliferation is absent in the adult fully differentiated mammalian myocardium. Moreover, experimental results of acute cardiac hypertrophy in rodents have demonstrated the inability of cardiac myocytes to re-enter the cell cycle (Chien and Olson, 2002). These observations have led to the dogma that, shortly after birth, ventricular myocytes withdraw from the cell cycle permanently, and are destined to die without further replication. This dogma implies that hypertrophy is the main, if not the only, form of myocyte growth in the heart after birth. In addition, the sporadic reports of DNA synthesis are considered insufficient proof of myocyte proliferation since in many species cardiac myocytes can become multinucleated and/or can undergo polyploidy (Clubb and Bishop, 1984; Li et al., 1996; Vliegen et al., 1991). The strongest argument in favour of new myocyte formation in the adult heart is the increase in myocyte number from birth to young adulthood in both animals and humans (Anversa and Nadal-Ginard, 2002). However, recent evidence shows that the new myocytes are not formed by proliferation of existing ones but arise from stem cells that transdifferentiate into cardiac myocytes if incorporated in host myocardium (Kajstura et al., 1998; Nadal-Ginard et al., 2003a; Nadal-Ginard et al., 2003b). Quaini et al. (Quaini et al., 2002) have demonstrated the presence of male myocytes in female hearts that were transplanted into male patients, thereby

illustrating the capacity of the heart to attract circulating stem cells that differentiate to cardiac myocytes.

Myoblasts, another type of differentiated cells, differ from neurons in the fact that they are often accompanied by precursor cells, termed “satellite cells”, which are normally dormant. Upon muscle damage, the rupture of myoblasts triggers the proliferation of the satellite cells. These will subsequently differentiate in myoblasts and form myotubes, thereby replacing the damaged tissue (Campion, 1984).

Ho and Hanawalt examined the repair of UV-induced CPD in the rat L8 myoblast cell line (Ho and Hanawalt, 1991) and demonstrated that as long as these cells proliferate they have a generally low repair capacity both at the global genome level and in transcribed genes. Upon terminal differentiation an initial decrease in global genome repair was observed, followed by a slow recovery. Gene-specific repair of active genes was improved by differentiation, but gene repair of silent genes became worse upon differentiation. In addition these cells did not demonstrate a strand bias for repair, indicating that DAR also exists in differentiated muscle cells.

Like neurons, adipocytes do not replicate after birth. The influence of terminal differentiation on UV-induced DNA damage and its repair in transcriptionally active and inactive genomic sequences can be investigated using the murine 3T3-T proadipocyte cell culture system. Actively cycling 3T3-T cells terminally differentiate into adipocytes after exposure to media containing platelet-depleted human plasma. Using this system it became clear that there is no heterogeneity in repair of UV-induced CPD (Bill et al., 1991). Transcribed and non-transcribed genes were repaired at similar rates in terminally differentiated adipocytes. Moreover, the rate of repair equalled the rate of repair observed in non-transcribed genes of undifferentiated stem cells.

Although they appear to be terminally differentiated, hepatocytes retain the ability to proliferate if the system is provoked by injury. For this reason one might predict that these cells are proficient in DNA repair and do not accumulate lesions in their untranscribed DNA. Indeed it has been demonstrated that primary cultures of non-dividing hepatocytes display TCR of UV-induced damage (Guo et al., 1998).

Double strand break repair

DNA double strand breaks (DSB) are frequently formed by exogenous and endogenous factors including ionizing radiation, oxidative damage of the

backbone, mechanical stress, cellular metabolizing agents, certain chemicals such as bleomycin and through the process of DNA replication itself. Efficient repair of DSB is necessary since replication and transcription are blocked at the site of a DSB. Moreover, the exposed ends are susceptible to degradation possibly leading to loss of genetic information. DSB are repaired via the following pathways (Karran, 2000).

Homologous recombination (HR) requires the presence of homologous DNA, either a homologous allele or a sister chromatid, as the genetic information on this intact DNA strand can be used as a template for DNA repair synthesis, thereby preventing the loss of genetic information. The phenomenon by which the DSB repair is carried out within one single chromosome, with the damage on one chromatid being eliminated using the other chromatid, is referred to as sister chromatid exchange (or sister chromatid conversion). In mammalian cells this is probably much more common than interchromosomal recombination (chromosome exchange or chromosome conversion). Although mitotic or allelic recombination would restore the original genomic physical organization it could result in loss of heterozygosity (LOH) (Bishop and Schiestl, 2003). Homologous recombination comprises the following steps (I) nucleolytic processing of the DNA ends to create single-stranded regions, (II) following the search for homologous duplex DNA, (III) DNA strand exchange generates a joint molecule between the homologous damaged and undamaged duplex, (IV) DNA synthesis and ligation, and (V) resolution of the recombination intermediates.

The most prominent repair process in somatic cells to cope with DSB repair is non-homologous end-joining (NHEJ). NHEJ requires limited processing of the ends, i.e. removal or addition of a few base pairs, after which the ends are joint and ligated. Since this process does not require any sequence homology in contrast to HR, it is error-prone (Valerie and Povirk, 2003).

Cell death

Cell death

Two major pathways of cell death are known that differ morphologically and biochemically. The first one to be described, necrosis, is a passive process that most often occurs following severe and acute damage of the cell. In necrosis early changes in mitochondrial function and shape occur and the cell rapidly becomes

unable to maintain cellular homeostasis. The major site of damage is the plasma membrane and loss of membrane integrity results in the dysregulation of osmotic pressure, leading to swelling and rupture of the cell. The cellular contents are released in the surrounding tissue space, thereby provoking an inflammatory response.

The second form of cell death is apoptosis. Although this physiologic form of cell death has been known for decades, the interest in this subject was renewed when Kerr (1972) described the ultrastructural changes of dying cells in detail and adopted the term apoptosis to describe the process. The word “apoptosis” (αποπτωσις) is used in Greek to describe the “dropping off” or “falling off” of petals from flowers, or leaves from trees, and in multicellular organisms the elimination of excess, damaged or harmful cells.

In contrast to necrosis, apoptosis is an active process in which a single cell initiates an inherent suicide program resulting in the successive fragmentation of the cell. Apoptotic cellular fragments are subsequently ingested by neighbouring cells, mostly macrophages (Fadok and Chimini, 2001; Henson et al., 2001). The whole process of cell fragmentation and digestion occurs without leakage of cellular contents in the environmental tissue space and hence does not cause any inflammatory response. The absence of inflammation permits cell death without damage to adjacent cells.

Apoptosis is an evolutionary conserved process to eliminate unwanted, damaged, aged or misplaced cells during embryogenesis and tissue homeostasis (Meier et al., 2000; Vaux and Korsmeyer, 1999). Abnormal resistance to apoptosis together with deregulated cell division can lead to disorders such as autoimmunity or cancer due to the persistence of superfluous or mutated cells (Hersey and Zhang, 2003; Ivanov et al., 2003). Enhanced apoptosis, on the other hand, may contribute to acute diseases such as infection by toxin-producing micro-organisms, ischemia-reperfusion damage or infarction as well as to chronic pathologies, such as neurodegenerative and neuro-muscular diseases and AIDS (Badley et al., 2003; Hou and MacManus, 2002; Ikeda et al., 2003; Rodriguez et al., 2002).

Morphology and biochemical characteristics of apoptosis

Apoptosis differs morphologically and biochemically from necrosis (reviewed by Cohen, 1994 and Ueda and Svah, 1994). In apoptosis, affected cells shrink in volume and lose microvilli and cell-cell contact. These changes occur rapidly and are accompanied by surface convolution. Subsequently the cell explodes into a

series of membrane-bound condensed apoptotic bodies. In contrast to necrosis, the morphology of mitochondria and ribosomes of apoptotic cells has normal structure and cytoplasmic organelles remain mainly intact during this whole process. The most outstanding morphologic and biochemical changes, however, occur in the nucleus. The nuclear events occurring during apoptosis include changes at the molecular level (i.e. DNA cleavage, modifications of nuclear polypeptides, and proteolysis of several proteins important for cell maintenance), and, consequently, alterations at the morphological level (i.e. chromatin condensation, nuclear shrinkage, and DNA fragmentation) (Rogalinski, 2002). The cell shrinks, losing as much as 30% of its volume, and the plasma membrane starts to ruffle and forms blebs but remains intact. The cell separates into a cluster of membrane bound segments, “apoptotic bodies”, which often contain morphologically normal mitochondria and other cell organelles. Soon after this process is initiated, a change in the cell surface occurs and apoptotic bodies are phagocytized by macrophages or other adjacent viable cells. Since there is no spillage of pro-inflammatory cell content, removal of cells by apoptosis will not cause a pro-inflammatory response.

Caspases

At the heart of the apoptotic pathway is a family of cysteine proteases, the caspases (cysteiny aspartate-specific proteinases). The name caspases is based on two catalytic properties of the enzymes. The “c” is intended to reflect a cysteine protease mechanism, and “aspase” refers to their ability to cleave after aspartic acid, being the most distinctive catalytic feature of this protease family (Alnemri et al., 1996).

Caspases are present in the cell as inactive pro-caspases that are cleaved and activated in response to apoptotic stimuli. The role of these enzymes in cell suicide is to disable critical homeostatic and repair processes as well as to cleave key structural components, resulting in the systematic and orderly disassembly of the dying cell (Nicholson and Thornberry, 1997). Polypeptides known to be cleaved during apoptosis include 1) enzymes involved in genome function, such as poly(ADP-ribose) polymerase (PARP) and the catalytic subunit of DNA-dependent protein kinase (DNA PK), which is involved in DSB repair, 2) regulators of cell cycle, including the retinoblastoma gene product and mouse double minute 2 protein, and 3) structural proteins of both the nucleus and the cytoskeleton, such as the lamins and actin. Finally, cells that are embedded in organized tissue structures

need to be detached, a process which is facilitated in part by the cleavage of FAK (van de Water et al., 2001).

These cleavage events are meant to: 1) halt cell cycle progression, 2) disable homeostatic and repair mechanisms, 3) initiate the detachment of the cell from the surrounding tissue structures, 4) disassemble structural components, and 5) mark the dying cell for engulfment by other cells.

Caspases can be divided into two categories: the initiator caspases which include caspase 2, 8, 9, 10 and 12, and the effector caspases which include caspase 3, 6 and 7. The initiator caspases are activated at an early point in the apoptotic signalling pathway. Once activated, the initiator caspases activate the effector caspases through cleavage at specific internal Asp residues, in order to amplify the signal. Effector caspases are the work horses as they cleave the substrates which are responsible for the dismantling of the cells in apoptotic bodies.

The “death-receptor pathway”

In mammals at least two distinct caspase-dependent apoptotic signalling pathways have been described. The first is called the “death-receptor pathway” and is initiated by extracellular death-inducing ligands of the tumor necrosis factor (TNF) superfamily, such as TNF α , FasL/CD95L, TWEAK and TNF related apoptosis-inducing ligand (TRAIL) (Krammer, 2000; Locksley et al., 2001; Schneider and Tschopp, 2000). These ligands bind to cell surface receptors (TNFR, Fas/CD95R, DR3, and DR4/DR5, respectively) leading to their trimerization. Via an intracellular domain, termed the death domain (DD), the trimerized death receptors recruit non-enzymatic adaptor proteins, such as TNF receptor-associated death domain (TRADD) and/or Fas-associated death domain (FADD). The initiator caspase 8 forms an interaction with this complex via the death effector domain (DED) and is activated by autoproteolysis due to the proximity of TRADD and/or FAD. Once activated, caspase 8 cleaves and activates down stream effector caspases (Kruidering and Evan, 2000).

Mitochondrial control of apoptosis

The second pathway to caspase activation is under the control of members of the Bcl-2 family and is termed the mitochondrial apoptotic pathway. This pathway is predominantly triggered by death-receptor independent stimuli, such as UV and γ

irradiation, chemotherapeutic agents, viruses, bacteria, and the detachment of cells from the ECM (anoikis). These stimuli target various intracellular components, which transmit the signal to the caspase machinery via specific sensors. In comparison to the execution phase of the mitochondrial apoptotic pathway, relatively little is known about how the upstream signalling pathways to the mitochondria are regulated. In particular, it is not yet elucidated how the signals from either developmental cues or damage signals are transduced to and integrated in the mitochondria. However, at some point the second messengers accumulate and converge to mitochondria, that subsequently undergo a membrane permeabilization (Wang, 2001). This mitochondrial membrane permeabilisation (MMP) causes a disruption of the mitochondrial transmembrane potential ($\Delta\psi_m$) and marks a point of no return in the apoptotic process (Kroemer, 2003). Due to the dissipation of the $\Delta\psi_m$, proteins (such as cytochrome c), that are hidden in the intermembrane space of healthy mitochondria, migrate to the cytoplasm and may contribute to both caspase-dependent and caspase-independent processes of apoptotic cell death (Ravagnan et al., 2002; van Gurp et al., 2003).

The Bcl-2 protein family

The Bcl-2 family of proteins integrates diverse survival and death signals that are generated inside and outside the cell and are therefore central regulators of apoptosis. The family is subdivided into two classes: anti-apoptotic members such as Bcl-2, Bcl-xL (the Bcl-2-like survival factors) which protect cells from apoptosis, and the pro-apoptotic members such as Bax and Bak (the Bax-like death factors) and the large group of BH3-only death proteins, which trigger or sensitize the cell for apoptosis (Borner, 2003). How the Bcl-2 family proteins regulate apoptosis is still an issue of debate. In mammalian cells the cytochrome-c release from mitochondria during apoptosis can be prevented by Bcl-2 (Hengartner, 2000). For this reason it is believed that Bcl-2 functions to preserve mitochondrial membrane integrity (Green and Reed, 1998; Gross et al., 1999; Wang, 2001). Upon an apoptotic stimulus the pro-apoptotic protein Bax moves from the cytosol to the mitochondria, where it forms clusters with Bak (Wang, 2001). According to one theory, Bax/Bak-like proteins form mitochondrial pores either directly or indirectly, and subsequently initiate the caspase cascade that occurs downstream the mitochondria (Green and Reed, 1998; Gross et al., 1999; Wang, 2001). Although the complexity of the apoptotic signalling process and the role of the Bcl-2 family proteins are still not completely understood, it is believed that the BH3-only death

factors “sense” the apoptotic stimuli and inhibit Bcl-xL/Bcl-2 and/or activate Bax/Bak. The activation of Bax/Bak leads to caspase-dependent and caspase-independent death signalling by mitochondrial membrane perforation. Bcl-xL/Bcl-2-like survival factors block these events by sequestering BH3-only and/or Bax/Bak (Borner, 2003).

Cell death and the heart

Apoptosis of cardiac myocytes has been described in a variety of cardiovascular diseases, including heart failure, ischemia without or with reperfusion, atherosclerosis, myocarditis, transplant rejection and arrhythmogenic disorders. The loss of cardiac myocytes in the heart means that these cells are lost forever. Therefore, apoptosis of cardiac myocytes at a low frequency will result in a gradual loss of functional cardiac myocytes and may, over years, lead to a substantial loss of pump function of the heart. Apoptosis may be an important process contributing to the progress of heart failure (Bing, 1994).

Myocyte cell death during ischemia and reperfusion

Until 10 years ago, necrosis was considered to be the only, or at least the main, mechanism of myocyte death after myocardial infarction. Cell death was secondary to energy deficit, cytoplasmic proteins were released and there was an inflammatory reaction by infiltrating macrophages, which preceded scar formation. Lately, however, experimental studies have shown that myocytes undergo cell death by necrosis as well as apoptosis during ischemia and reperfusion. Whether apoptosis is triggered during ischemia or during reperfusion is still controversial. In the 1990's evidence grew that apoptosis precedes necrosis and constitutes the prevailing form of myocyte death. Apoptosis of cardiac myocytes appears to be the major form of initial myocardial damage produced by occlusion of a major epicardial coronary artery. Necrotic cardiac myocyte death mostly followed apoptosis and contributed to the progressive loss of cells in the infarcted region of the wall (Bardales et al., 1996; Kajstura et al., 1996). Recent evidence indicates that apoptosis is primarily expressed during reperfusion (Fliss and Gattinger, 1996; Gottlieb et al., 1994; Zhao et al., 2000) and that necrosis rapidly develops after ischemia, progressing from subendocardial to subepicardial regions

of the left ventricular wall. Without reperfusion necrosis reaches its full transmural extent after 6-7 hours of ischemia (Rochitte et al., 1998; Zhao et al., 2000). However, infarct size may be determined not only by the duration of ischemia but also by pathological events occurring during reperfusion. Necrosis appears to be a dynamic pathologic process that continues over at least 24 hours of reperfusion after a fixed period of ischemia (Rochitte et al., 1998). In contrast to the extent of necrosis, which peaked at 24 hours of reperfusion the appearance of apoptotic cells in the peri-necrotic area progressively increased up to 72 hours of reperfusion (Zhao and Vinten-Johansen, 2002).

It is not clear yet whether apoptosis precedes or is followed by necrosis, or whether both mechanisms of cell death occur simultaneously by separate pathways. The data suggest that necrosis and apoptosis occur simultaneously during reperfusion, with necrosis developing relatively rapid, during the early phase of reperfusion and apoptosis following during the late phase of reperfusion. However some studies suggest a crossover of apoptosis to necrosis (Umansky et al., 1997; Umansky and Tomei, 1997). Other investigators have shown that infarct size in an in vivo rat model is reduced by inhibiting apoptosis in the acute stage (Vakeva et al., 1998; Yue et al., 1998).

Myocyte cell death after myocardial infarction

In patients with a history of myocardial infarction heart failure may develop in the absence of ongoing ischemia or recurrent infarction. Loss of contractile function in this group of patients is attributed to post-infarction LV remodelling. Three regions are evident in the infarcted heart: infarcted myocardium which is unperfused and akinetic; borderzone myocardium, which is perfused and hypokinetic, and the so-called remote myocardium, which is well perfused and has preserved contractility. Immediately after infarction the extent of borderzone myocardium may be small, and function may be impaired only moderately. However, in time the area of dysfunctional myocardium may become enlarged leading to progressive impairment of function (Jackson et al., 2002). Although the exact cause of the progressive reduction in contractility of uninfarcted borderzone myocardium is not established yet, increased stress and the resulting strain may fundamentally alter the borderzone myocardium. Experimental studies have demonstrated that expanding regional infarction can initiate a myopathic process that spreads beyond the immediate peri-infarct region and may involve the entire ventricle (Jackson et al., 2002). Apoptotic myocytes are found in the borderzone myocardium (Jackson

et al., 2002) suggesting that non-ischemic myocyte loss due to stretch-induced apoptosis produces global ventricular dysfunctioning.

Myocyte cell death in failing hearts

Evidence is growing that myocyte cellular degeneration is one of the most prominent phenomena in failing human myocardium. Myocytes die by multiple mechanisms in the failing human heart: apoptosis, oncosis and autophagic cell death (Narula et al., 1996; Olivetti et al., 1997; Kostin et al., 2003). Although there is no doubt that apoptosis plays an important role in cardiac diseases, the importance of apoptosis still has to be clarified. The problem of quantification of apoptotic cells has not been completely solved because of the following three reasons: methodological (overinterpretation of results, no differentiation between myocytes and non-myocytes), experimental (global or regional acute ischemia, chronic conditions such as heart failure or hibernating myocardium), and interpretation (unknown time period for the completion of apoptosis). This problem is reflected in the large differences in incidence of apoptosis reported (Elsasser et al., 2001; Freude et al., 1998).

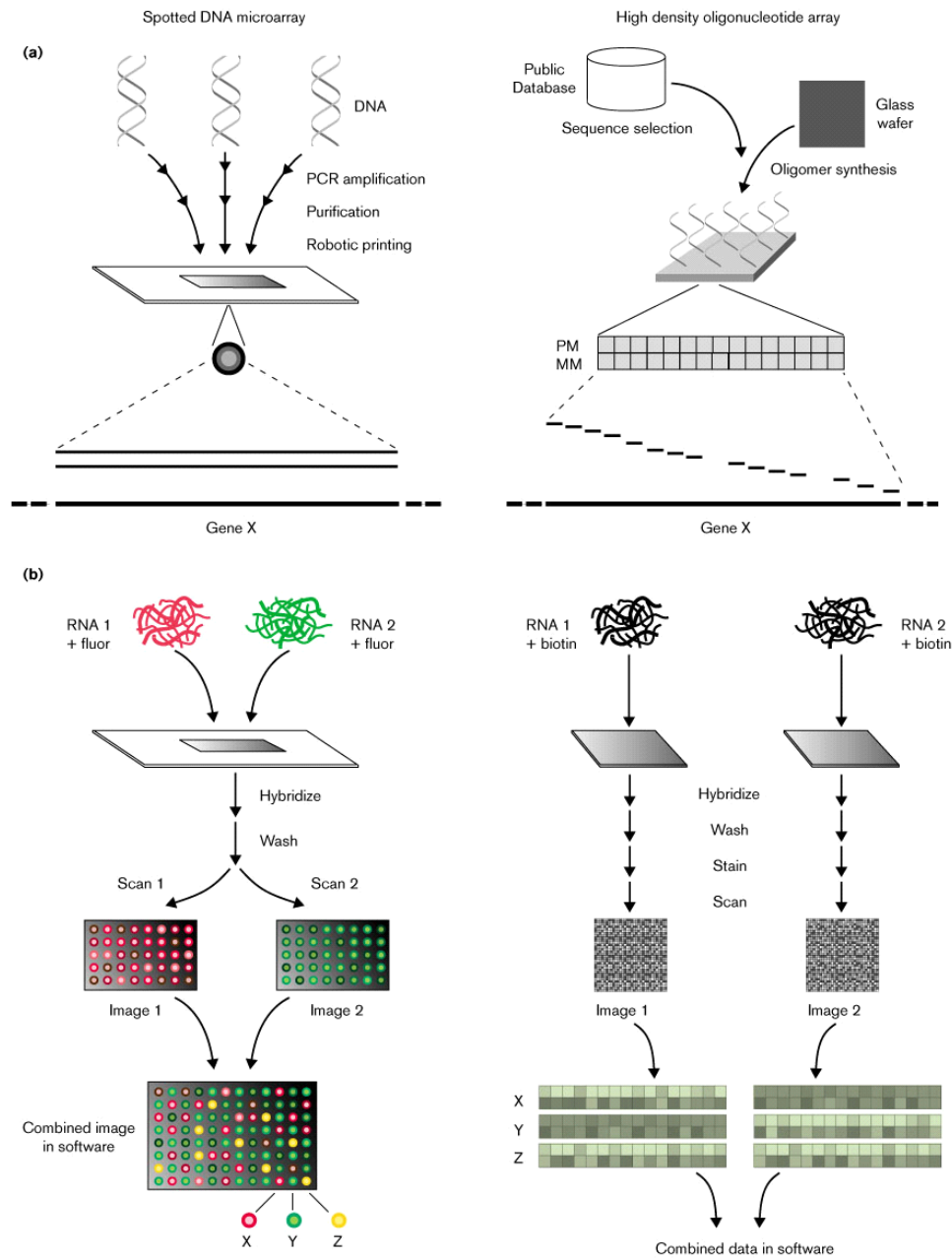
Coronary artery disease is the etiologic factor in 68% of the patients with heart failure (Gheorghiade and Bonow, 1998), indicating that two third of the cases suffers from myocardial ischemia. Ischemia and heart failure associated neurohumoral activation of the heart by the sympathetic nervous system and the renin-angiotensin system create environmental stresses to the heart which are exaggerated by progressive stretch of the ventricular walls.

Micro array analysis

Micro arrays

In a living organism thousands of genes and their products (i.e. RNA and proteins) function in a complicated and orchestrated way. Traditional methods in molecular biology research generally investigate on a “one gene in one experiment” basis, which means that the throughput is very limited and the “whole” picture of gene function is hard to obtain. In the past several years, a new technology, termed DNA micro array, has attracted tremendous interest among biologists. DNA micro

array allows the monitoring of the whole genome on a single chip thereby creating a better picture of the interactions among thousands of genes simultaneously.



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Figure 5. Schematic overview of the two variants of micro arrays. See text for detailed information.

A micro array is a solid support, either a glass slide or a membrane, on which oligonucleotide or cDNA probes are spotted in an orderly fashion. Sample RNAs obtained after experimental treatment are radioactively or fluorescently labelled, and subsequently hybridised to the arrays. The intensity of each spot, which appears after hybridization, is a measure of the abundance of the RNA transcript that has hybridised to the spot.

There are two variants of micro arrays (figure 5). In the first one, traditionally called DNA micro array, cDNA probes are immobilized to a solid surface using robot spotting. These DNA micro arrays are exposed to a set of targets, either separately or in a mixture. In case of exposing the DNA micro array to a set of targets, control and experimental target RNAs are labelled with a different dye, usually cy3 (red) and cy5 (green). The ratio of the red to the green signal gives information about the relative abundance of the transcript in both the experimental and the control sample.

The second type of micro array, the DNA chip, is created by synthesizing a series of oligonucleotide (20~28-mer oligos) or peptide nucleic acid (PNA) probes, either in situ (on the chip) or by conventional synthesis followed by on-chip immobilization. This array is exposed to a labelled RNA of one sample. Afterwards several arrays can be compared by computer analysis.

The study described in this thesis uses oligonucleotide arrays (RG-U34A) of Affymetrix (Santa Clara, CA) to analyse gene expression in rat cardiac myocytes and fibroblasts after in vitro exposure to mechanical stress as compared to control conditions (no stress).

GeneChip array design

The oligonucleotides for the high density oligonucleotide GeneChip arrays from Affymetrix are designed based on the sequence of the gene of interest. Using a photolithographic reaction (Wodicka et al., 1997) the oligonucleotides are synthesised in situ on the glass support. Each oligonucleotide containing element on the array is termed a probe cell (figure 6).

On the GeneChip arrays, designed for the rat, an expressed transcript is represented by a probe set that consists of 16 unique probe-pairs, with no or minimal overlap. Each probe pair is composed of a perfectly matching probe (PM) of 25 oligonucleotides and a "mismatching" counterpart (MM), which is a copy of the PM, except that the thirteenth nucleotide is replaced by a mismatching nucleotide (figure 6).

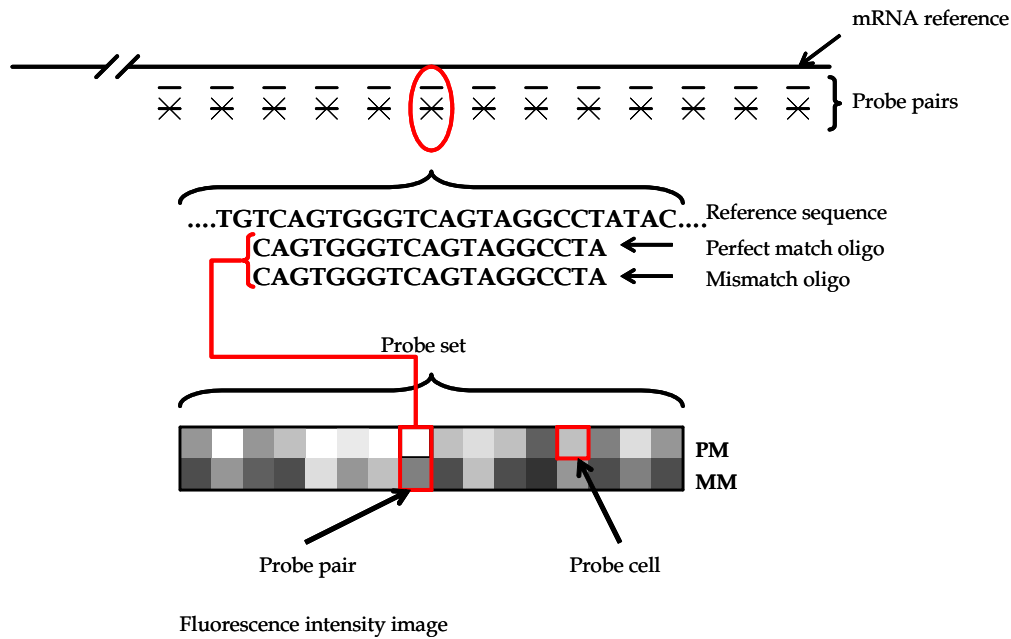


Figure 6. Schematic representation of the design of a GeneChip. See text for detailed information. PM: perfect match oligo, MM: mismatch oligo.

The reason for including a MM probe is to provide a value that comprises most of the background hybridization and stray signal affecting the PM probe and also for probe-specific fluorescence features. It is known, for instance, that probes with many C or G have different fluorescence features than probes with many A or T (Hubbell et al., 2002).

At present, opinions differ on whether to subtract the signal of the MM probe from the signal of the PM probe, or whether it is better to only use the PM signals in data analysis. In former studies, the means of PM-MM signals of a probe-set was shown to be directly related to mRNA abundance (Lipshutz et al., 1999; Lockhart et al., 1996; Parsons et al., 2003). On the other hand, Chu et al. (Chu et al., 2002) showed that the effect of taking MM-values into account had little effect on the final outcome of their model.

To monitor the transcriptional response of cardiac cells to the different stresses only the PM signal was used. To describe the signal of every PM probe a linear model based on a method described by Chu et al (2002) was used. As described before, micro arrays from Affymetrix use a probe set consisting of multiple different probe pairs for each transcript to estimate the quantity of specific mRNA

molecules. By analysing data with the standard software (MAS 5.0) the signals of these probe pairs are usually averaged to obtain an estimate of RNA concentration for each gene. The estimates are subsequently used to determine fold changes, calculate signal to noise ratios and to cluster and classify data. Especially when expression level differences are small or within the range of background noise, the discrepancy between true RNA levels and the estimated concentrations is a source of frustration. In contrast to MAS 5.0, the linear model used in our studies uses individual probe signals instead of estimates throughout the whole analysis, resulting in a sensitive and accurate method to detect changes. Another major improvement is the opportunity to combine several micro arrays belonging together to a meta-array.

Micro array analysis of expression patterns in cardiac cells

Myocardial tissue has a repertoire of methods to adapt to potentially harmful conditions. Several conditions may be considered beneficial on a short term, but may become detrimental on a long term. At the molecular level, the functional adaptations involve quantitative and qualitative changes in gene expression. Quantitative changes may involve expression of constitutively expressed genes. Qualitative changes in gene expression may include positive and negative modulation of cardiac-specific genes and a shift in the expression of several genes towards a fetal gene repertoire.

Until now little is known about gene expression patterns in cardiac cells after a period of mechanical stress. In previous studies, the changes in in vitro gene expression after exposure to mechanical stress were examined by Northern blotting. A major disadvantage of this method is that a limited number of genes can be investigated, which are pre-selected by the researcher. Micro arrays give the opportunity to evaluate thousands of genes simultaneously in one assay. Gene expression profiling provides information about the dynamics of total genome expression in response to environmental changes and may point to candidate genes responsible for the cascade of events that result in disease. By using this technique new mechanical stress responsive genes might be determined.

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**Mechanical overload-induced apoptosis: a
study in cultured neonatal ventricular
myocytes and fibroblasts.**

Chapter two

Mechanical overload-induced apoptosis: a study in cultured neonatal ventricular myocytes and fibroblasts.

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Molecular and Cellular Biochemistry 241: 115-124, 2002

Abstract

Apoptosis of cardiac myocytes has been implicated in cardiac dysfunction due to chronic hemodynamic overload. Reports on the role of apoptosis in the transition from hypertrophy to decompensated heart failure are not unequivocal. In this study we analysed the direct relationship between mechanical overload and induction of apoptosis in an in vitro model of cultured heart cells.

Cyclic mechanical stretch was applied to cultured neonatal rat ventricular myocytes and fibroblasts. Several indicators of apoptosis were examined, such as morphological features, caspase-3 activity and DNA fragmentation.

Mechanical strain did not induce any significant change in these parameters as compared to non-stretched myocytes or fibroblasts. However, administration of staurosporine, a known inducer of apoptosis, induced massive apoptosis in myocytes as well as fibroblasts.

We conclude that this in vitro cell model system lacks a direct link between mechanical stretch and apoptosis. The three-dimensional structure-function relationship of myocardial tissue in the intact heart may elicit stretch-induced molecular signaling cascades in a much more complex way than in monolayer cultures of cardiac cells.

Key words

mechanical stretch, apoptosis, neonatal rat heart cells.

Introduction

Excessive pressure or volume load of the heart, as seen in hypertensive and valvular heart disease, results in an adaptive growth response leading to structural and functional cardiac changes. This cardiac remodelling includes enlargement of cardiac myocytes and an increase in fibroblasts and interstitial fibrosis to compensate for the increased workload (Swynghedauw, 1999). If these adaptations and functional alterations are insufficient to normalize the wall stress, transition from compensated hypertrophy to heart failure may occur (Bing, 1994; Colucci,

1997). Various factors have been considered to be involved in this process that is triggered by increased mechanical stress, such as activation of cytokines, oxidative stress and abnormalities in calcium homeostasis (Givertz and Colucci, 1998). Clinical as well as experimental evidence for a role of stress-activated cytokines in chronic heart disease have been reported: TNF α levels are progressively elevated in patients with increasing severity of heart failure (NYHA Functional Class) (Baumgarten et al., 2000). Also, increased levels of reactive oxygen species were detected in patients with heart failure (Keith et al., 1998), whereas activation of stretch-activated ion channels and prolongations in Ca²⁺ transients induce changes in contractile function (Zhang et al., 2000).

These factors may all be responsible for (ir)reversible myocardial injury and may even lead to loss of cardiac cells by apoptosis (Narula et al., 1999; Sabbah, 2000). A decrease in the number of myocytes may contribute to impaired contractility of the whole heart, but whether this is the primary cause or the result of the progressive cardiovascular dysfunction remains unclear (Elsasser et al., 2000).

The present study investigates the direct effect of mechanical overload on the induction of apoptosis in cardiac myocytes and fibroblasts. Our hypothesis is that the increased mechanical stimulus is sensed by specific mechanoreceptors, transduced into the cell where it could, directly or indirectly, activate the apoptotic pathway (Tavi et al., 2001). For this purpose neonatal rat ventricular myocytes and fibroblasts were cultured on plates with deformable growth surfaces and subjected to pulsatile stretch. In previous studies we used this model to determine the hypertrophy-related changes in gene expression and signal transduction in response to stretch (Ruwhof et al., 2001; van Wamel et al., 2000). It was demonstrated that stretch induces the release of factors that can initiate, in an autocrine or a paracrine way, a growth response in heart cells. In continuation of these experiments we focused on the long-term effect of increased mechanical strain on cardiac myocytes and cardiac fibroblasts. The stretch-induced deformation of the cells could result in apoptosis due to direct alterations in signal transduction pathways and changes in gene expression.

To prevent an unintentional interfering effect of culture conditions (low or high serum concentrations) on induction or suppression of stretch-induced signals leading to apoptosis, the cell viability of myocytes and fibroblasts under serum-deprived conditions was investigated first.

Materials and Methods

Cell cultures

Primary cultures of cardiac myocytes and fibroblasts were prepared from the ventricles of 2-day old Wistar rats as described previously (Persoon-Rothert et al., 1992). Briefly, ventricles were minced and cells were isolated enzymatically using collagenase type 1 (Worthington Biochemical Corp.) during two periods of 30 min in a shaking water bath at 37°C.

Pooled cell suspensions were pelleted by centrifugation, resuspended in growth medium consisting of Ham's F-10 supplemented with 10% (v/v) fetal bovine serum (FBS), 10% (v/v) horse serum (HS) (all purchased from Gibco, Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin and seeded in primaria-coated culture dishes (Falcon, Becton Dickinson). During a 45 min preplating step, in which fibroblasts attach at a higher rate to the culture dishes than myocytes, myocyte-enriched fractions were obtained. These preparations were seeded in 6-well Flex I plates with a flexible silicone elastomer bottom coated with collagen I (Flexcell International Corp.). Myocytes were allowed to attach, after which the growth medium was replaced by DMEM / Ham's F-10 medium (1:1) supplemented with 5% HS, 100 U/ml penicillin and 100 µg/ml streptomycin. Fibroblasts, attached to the primaria-coated culture dishes during the preplating step, were cultured on DMEM (Gibco, Life Technologies) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. After three days fibroblasts were trypsinized and also seeded in 6-well, collagen I-coated Flex plates and cultured for three days.

Cells were cultured in a humidified incubator with 5% CO₂ at 37°C.

Twenty-four hours before exposure to experimental treatments (stretch, staurosporine (Sigma)), the culture medium was changed to DMEM / Ham's F-10 medium (myocytes) or DMEM (fibroblasts), each containing 2.5% serum (HS or FBS, respectively) and antibiotics.

Mechanical Strain Unit

After 24 hours of low serum (2.5%) incubation, myocytes or fibroblasts cultured in 6-well Flex I culture plates were subjected to pulsatile stretch. Therefore, the plates were placed in the Flexercell Strain Unit FX-2000 (Flexcell) in which the frequency and magnitude of stretch are regulated by a computer-controlled vacuum pump.

The apparatus applied an equiaxial cyclic stretch of 20% elongation to the wells at a frequency of 60 cycles/min (1 Hz). Stretch was applied for various periods of time. Control cells were grown in identical culture plates and incubated in the same incubator as the stretched cultures, but were not mounted in the Flexercell Strain Unit.

Cell death

Cell death was quantified by the release of α -hydroxybutyrate dehydrogenase activity (α -HBDH, representing the cardiac LDH-1 isoenzyme activity) from the myocyte cultures and lactate dehydrogenase (LDH) release from fibroblast cultures using test kits (Roche). Enzyme activity was measured in the incubation medium and in aliquots of cell homogenates prepared in PBS containing 0.1% (w/v) Triton X-100.

Caspase-3 activity

Caspase-3 activity in myocytes and fibroblasts was measured by the cleavage of the fluorogenic Ac-DEVD-7-amino-4-methylcoumarin (DEVD-AMC) conjugate (BIOMOL) according to van de Water et al. (van de Water et al., 1999). Briefly, cells were collected by scraping, pelleted by centrifugation, resuspended in lysis buffer (10 mM HEPES, 40 mM β -glycero-phosphate, 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, pH 7.0) and subjected to three cycles of freezing and thawing.

Cell lysates were centrifuged for 30 min at 13000 rpm and the protein concentration of the supernatants (cytosolic extracts) was measured with a BCA Protein Assay (Pierce).

Cell lysate, containing 40 μ g protein, was combined (in duplicate) with 25 μ M DEVD-AMC and dissolved in reaction buffer (100 mM HEPES, pH 7.25, 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40, 10 mM DTT) to a total volume of 100 μ l. DEVD-AMC hydrolysis was measured fluorometrically in 96-wells white clear bottom plates (Costar) with a Victor 1420 multilabel counter (WALLAC). Changes in fluorescence over a 1 hour period were recorded every 10 min by exciting at 355 nm and measuring emission at 460 nm, using several concentrations of 7-amino-4-methylcoumarin (AMC, ICN Biomedicals) for fluorescence calibrating purposes.

The caspase-3 activity was calculated from the linear portion of the fluorescence versus time relation and expressed as pmol AMC / min / mg protein.

Ultrastructural features

To detect changes in cell viability in control and stretched cells, myocytes and fibroblasts were stained with Hoechst 33342 (10 µg/ml), and propidium iodide (5 µg/ml) during 10 min in the dark (both from Molecular Probes).

The cells were washed and viewed under a fluorescence microscope. The condensed chromatin structure of apoptotic cells was visualized by Hoechst 33342 staining of the nucleus. Propidium iodide stained nuclei of necrotic cells and nuclei of cells that underwent secondary necrosis following apoptosis.

DNA gel electrophoresis

DNA from control, stretched or staurosporine treated myocytes and fibroblasts was isolated to detect oligonucleosomal fragmentation. Five µg DNA was treated with 1 µl RNase (10,000 U/ml) for 1 h at 37°C. The DNA was mixed with 1 volume of neutral loading buffer (40% sucrose, 0.05% bromophenol blue) and electrophoresis was performed in 2.0% agarose at 75 Volt for 2 h in the presence of ethidium bromide to detect DNA laddering. The 100 bp molecular ruler (Biorad) was used as a reference.

Quantitative DNA fragmentation

The DNA fragmentation assay was performed as described by Kamesaki et al. (Kamesaki et al., 1993). This assay is based on the extraction of DNA fragments from Triton permeabilized cells. For this purpose, cultures on stretch plates were prelabeled with ³H-thymidine (1.0 µCi/ml, 111 Ci/mmol) for 24 hours at 37°C. Then cells were rinsed two times with PBS and fresh culture medium supplemented with 2.5% serum was added. After various time periods of stretch, the medium was collected and the cells were permeabilized in 250 µl lysis buffer, containing 5 mM Tris-HCl pH 8.0, 20 mM EDTA and 0.5% Triton X-100, on ice for 10 min. After centrifugation at 13000 rpm for 30 min at 4°C, supernatant and pellet were separated. The pellet was resuspended in 250 µl 0.2 N NaOH. The NaOH concentration of the culture medium and cell supernatant was adjusted to 0.2 N by addition of appropriate amounts of 10 N NaOH. All samples were incubated for 1 h at 65°C and ³H-counts were measured by a scintillation counter. DNA fragmentation was determined as: (cpm medium + cpm supernatant) / (cpm medium + cpm supernatant + cpm pellet + cpm bottom Flex plate).

Statistical analysis

Data represent means $\pm$ SEM of at least three independent experiments. Statistical significance was tested by one way analysis of variance (ANOVA) with $p < 0.05$ as the level of significance.

Results

Effect of serum deprivation on cell viability

A high concentration of serum components in the culture medium could suppress cell death signal transducers, while serum deprivation is known to induce apoptosis (Bialik et al., 1999).

Therefore, we first tested the viability of the cardiac myocytes and fibroblasts under different culture protocols. Standard serum conditions (5% HS for myocytes, 10% FBS for fibroblasts) were compared with incubations in 2.5% serum containing medium and serum-free medium for 24 hours (fig.1).

The release of the cytoplasmic α -HBDH activity from cardiomyocytes and LDH activity from fibroblasts was determined in the culture medium to assess necrotic cell death (fig. 1B). Furthermore, the activation of caspase-3, a cysteine protease activated during apoptosis, was measured in myocytes and fibroblasts cultured under standard, low and serum-free conditions (fig. 1C) and compared with the amount of protein per petri dish (fig. 1A).

These experiments showed a cell-specific response upon changes in serum concentrations. In the absence of serum, a decrease in the total amount of protein per petri dish was seen in both cell types (fig. 1A). Necrotic cell death in myocytes, as reflected by α -HBDH release, is partly responsible for this phenomenon (fig. 1B), as caspase-3 activity is almost unchanged (fig. 1C). However, in fibroblasts, a strong increase in caspase-3 activity is observed during serum-free incubations (fig. 1C), but no serum-dependent changes in LDH activity are visible (fig. 1B).

Thus, a progressive increase in cell death due to serum deprivation is observed in these cultures: in myocytes mainly caused by necrosis and in fibroblasts by apoptosis.

For the experimental protocol of the subsequent stretch study, the culture medium of myocytes and fibroblasts was changed to medium containing 2.5% serum, 24 hours before the start of the stretch experiments.

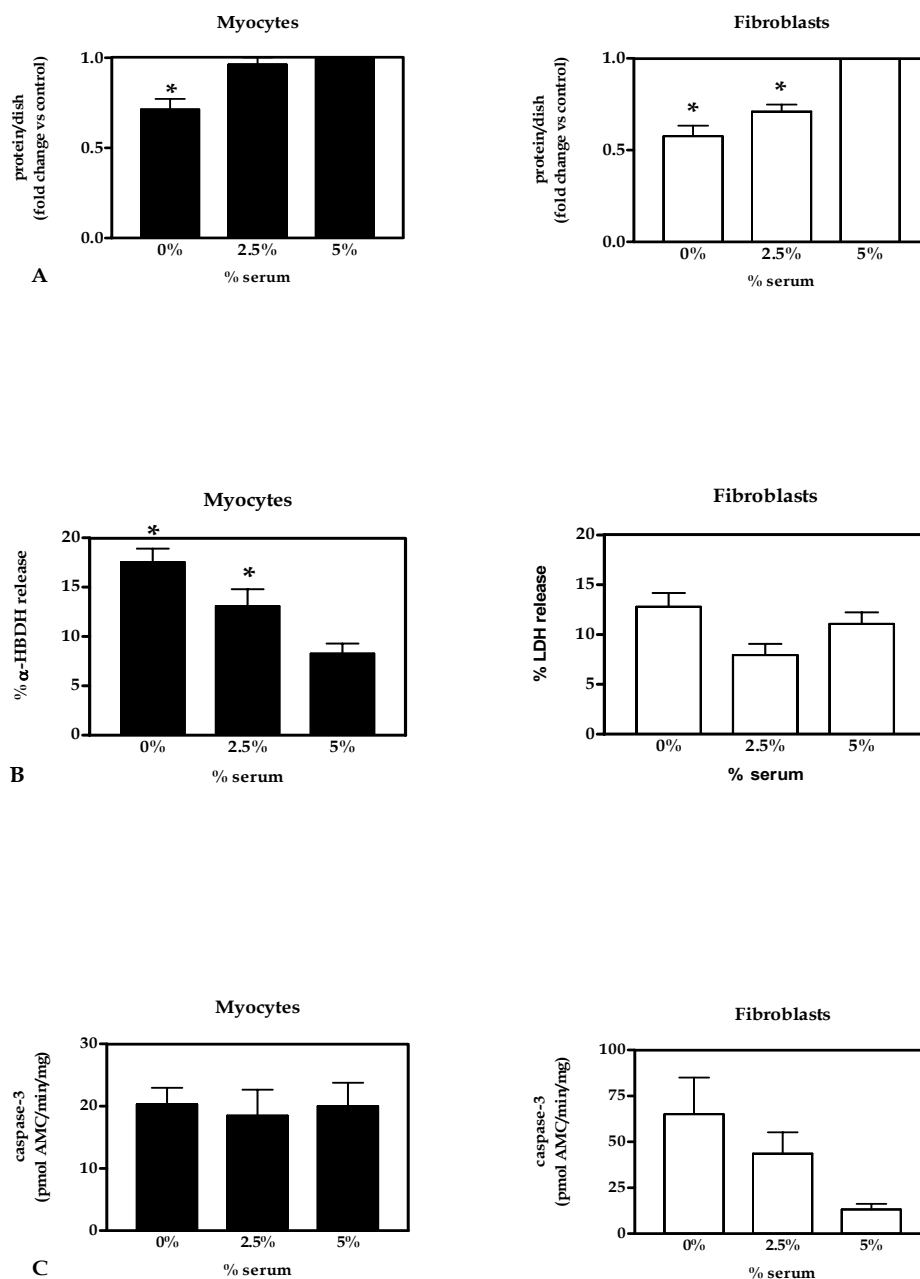


Figure 1. Serum deprivation induced changes in A. protein amount per petri dish (expressed as fold change versus standard medium), B. percentage α -HBDH release from myocytes and percentage LDH release from fibroblasts, and C. caspase-3 activity (expressed as pmol AMC formed per min per mg protein). Cells were grown on Flex I plates (no stretch) and incubated in 0, 2.5 and 5% (myocytes) or 10% (fibroblasts) serum containing medium for 24 hours. Means $\pm$ SEM, $p < 0.05$ compared to standard serum conditions: A: myocytes $n=4$, fibroblasts $n=3$, B: $n=5-11$, C: myocytes $n=4$, fibroblasts $n=3$.

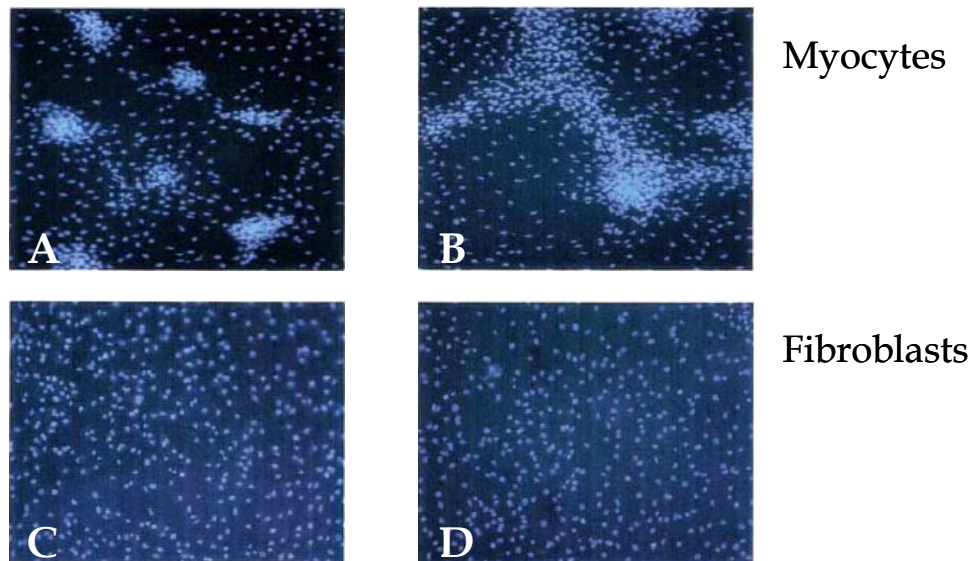
Effect of cyclic stretch on cell viability

Cardiac myocytes and fibroblasts were cultured on 6-wells plates with a flexible silicone elastomer bottom and subjected to a cyclic (1Hz) 20% stretch protocol to detect a direct effect of mechanical stress on the induction of apoptosis.

Morphology

The application of cyclic mechanical stress for 24 hours resulted in certain morphological changes in the monolayer of cells as displayed by low magnification of Hoechst 33342 stained nuclei (fig. 2). Myocytes adapted the increased mechanical strain by the formation of cell 'clusters' with a bundle-like structure (fig. 2 A, B), whereas in cardiac fibroblast cultures no such changes were seen (fig. 2 C, D). In stretched as well as in non-stretched cells no indices of necrosis (propidium iodide staining) or apoptosis (Hoechst 33342 staining) exceeding those of the control cell populations were observed.

Staurosporine, a known protein kinase inhibitor and inducer of apoptosis, was used in this study as a positive control. Addition of 500 nM staurosporine resulted in ultrastructural changes in both myocytes and fibroblasts.



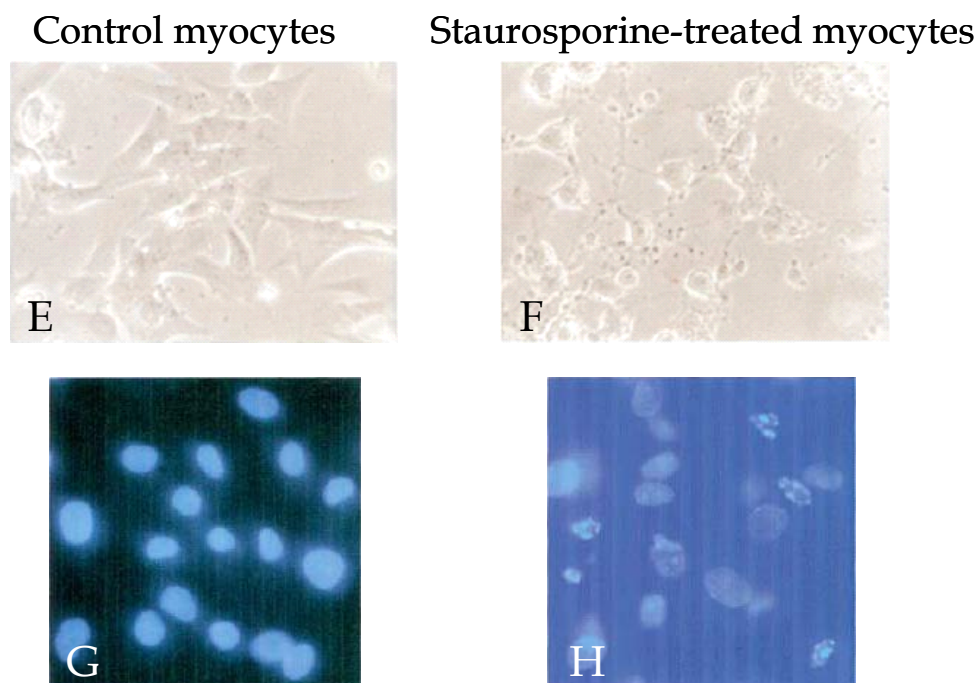


Figure 2. Overview of the Flex I culture plates with control (A) and stretched (B) myocytes and control (C) and stretched (D) fibroblasts. Stretch was applied for 24 h at 20% elongation, 60 cpm. Nuclei were stained with Hoechst 33342. Microscopic images of control (E) and 500 nM staurosporine-treated (F) myocytes after 24 h incubation. Control nuclei (G), visualized by Hoechst 33342 staining, and apoptotic nuclei after incubation for 6 h with 500 nM staurosporine (H). (Microscopy: magnification A - D x 100; E,F x 320; G,H x 400). The cells exhibited apoptotic features such as cell shrinkage, bleb formation (incubation time > 2 h) (fig. 2 E, F), nuclear condensation (fig. 2 G,H) and finally detachment from the growth surface at times > 6 hours.

Necrotic cell death

The release of α -HBDH activity from myocytes and LDH activity from fibroblasts into the culture medium was determined in cells subjected to stretch. In comparison to non-stretched cells, neither myocytes nor fibroblasts showed considerable enzyme release during experiments in which 24 hours of cyclic stretch (20%, 60 cpm) was applied.

In myocytes the percentage α -HBDH released from control cells was 11.9 ± 3.3 % versus 10.5 ± 0.9 % in stretched cells (n.s.). In non-stretched fibroblasts 7.6 ± 0.7 % LDH was released into the culture medium and in stretched cells 6.4 ± 0.8 % (n.s.).

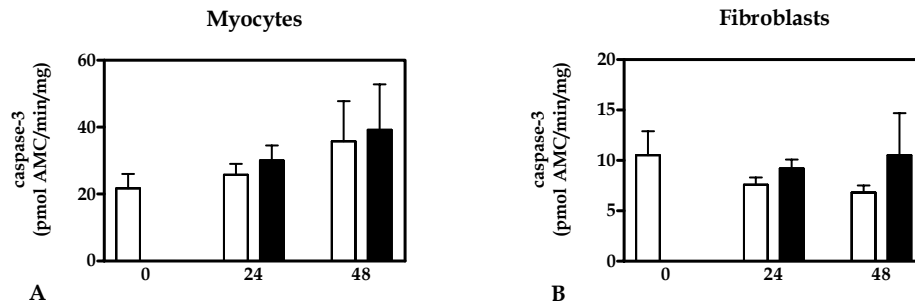


Figure 3. Caspase-3 activity (pmol AMC/min/mg protein) in stretched (60 cpm, 20% elongation, black bars) and control (open bars) myocytes and fibroblasts. Incubation time 0-48 hours. Means $\pm$ SEM, $n=3-8$.

Caspase-3 activity

After various time periods of mechanical stress the myocytes and fibroblasts were screened for changes in activity of caspase-3. Cells were stretched 0, 24 and 48 hours in a cyclic stretch protocol (60 cpm, 20%), whereafter the intracellular caspase-3 activity was measured (fig. 3 A,B). Control, non-stretched cells were grown in parallel. The caspase-3 activity was not statistically different between stretched and non-stretched cells. The pattern of caspase-3 activity in myocytes equals that in fibroblasts and increases slightly with time, probably due to ageing of the cultures. Even prolonged incubations up to 96 hours of stretch or application of stretch under serum-free conditions, did not reveal significant changes in caspase-3 activity between control and stretched cells (data not shown).

However, addition of 500 nM staurosporine resulted in a massive apoptotic response with a peak activity in caspase-3 activity after 4-6 hours in both myocytes and fibroblasts (fig. 4 A,B). A difference occurred in the specific activity of this protease between myocytes and fibroblasts grown on primaria-coated plastic culture dishes: per mg protein the maximal caspase-3 activity was approximately 1200 pmol AMC formed per minute in myocytes and 600 pmol AMC per minute in fibroblasts (fig. 4 A,B). This cell-type specific difference was also reflected in control cultures when grown on stretch plates at $t=0$: basal caspase-3 activity in myocytes was approximately 20 pmol AMC/min/mg protein versus 10 pmol AMC/min/mg in fibroblasts (fig. 3 A,B). Compared to the staurosporine data the stretch-induced changes in caspase-3 activity are only marginal.

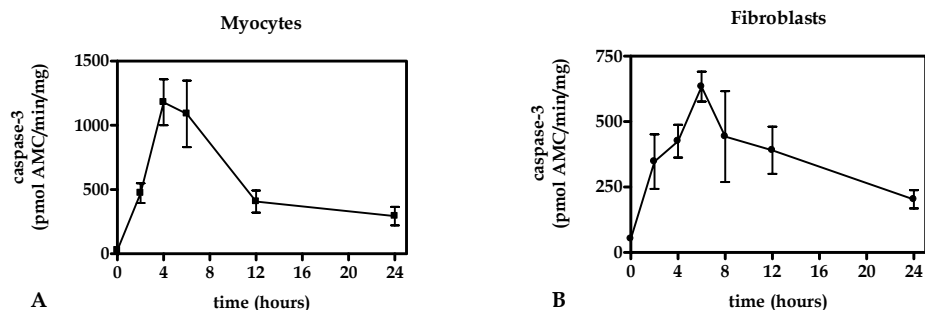


Figure 4. Time course of 500 nM staurosporine-induced caspase-3 activity (pmol AMC/min/mg protein) in myocytes (A) and fibroblasts (B). Means $\pm$ SEM, $n=3-10$.

DNA fragmentation

Administration of 500 nM staurosporine induces nuclear condensation and subsequent DNA fragmentation. After 24 hour incubation, DNA was isolated from floating and adherent myocytes separately and subjected to agarose gel electrophoresis. The staurosporine-treated cells clearly showed oligonucleosomal DNA fragmentation. No DNA ladder was detectable in adherent and floating cells of control and stretched cells (24h, 20% elongation, 60 cpm). In these lanes only unfragmented high molecular weight DNA was visible (data not shown). Comparable pictures were made from control, stretched and staurosporine treated fibroblasts (data not shown).

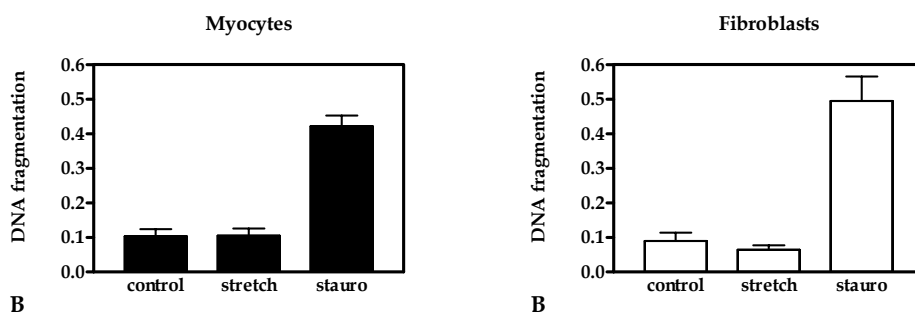


Figure 5. Quantitative DNA fragmentation in control, stretched (20% elongation, 60 cpm) and 500 nM staurosporine treated myocytes (A) and fibroblasts (B) during 24h. After prelabeling with ^3H -thymidine for 24 h, cells were stretched or treated with staurosporine for 24 h, followed by cell fractionation and ^3H -counting. The number of soluble ^3H -counts, expressed as a fraction of total ^3H -counts in the cell system is represented on the Y-axis. Means $\pm$ SEM, $\star:p < 0.05$ compared to control cells: myocytes $n=4$, fibroblasts $n=3$.

In addition a DNA fragmentation assay was used to detect the quantitative release of DNA from the cells, independent of fragment size. Also in this experiment only

staurosporine induced a substantial amount of DNA fragments in comparison to stretched and non-stretched myocytes and fibroblasts (fig. 5 A,B).

Discussion

Chronic mechanical overload leads to an increase in left ventricular mass and volume. Evidence accumulates that this response to overload includes apoptosis of cardiomyocytes, probably causing the transition to heart failure (Bing, 1994; Sabbah, 2000). To dissect the specific contribution of stretch in the multifactorial process of heart failure, an in vitro model system of stretch in cultured heart cells seems appropriate. However, the results from the present study do not support the view of stretch-induced apoptosis in cultured cardiac myocytes and fibroblasts. This conclusion is based on observations of cellular and nuclear morphology, proteolytic activity of caspase-3, and fragmentation of DNA. All stages of the apoptotic machinery, however, can be recognized in myocytes and fibroblasts, as was shown by the response upon staurosporine administration. Although our data indicate that increased mechanical stretch does not directly activate apoptosis, several considerations may limit the validity of extrapolations to the overloaded heart. Did the experimental model in which we looked for apoptotic processes represent the overloaded myocardium in this respect, and/or was the incidence of apoptosis below the level of detection?

First, the amount of stretch applied to cardiac cells must be representative for the in vivo pathophysiological situation in humans. Butt and Bishop (Butt and Bishop, 1997) and Ono et al. (Ono et al., 1995) reported that the degree of deformation applied by the Flexercell Strain Unit is appropriate for the mechanical tension experienced by the heart in vivo, whereas Shyu et al. (Shyu et al., 2001) stated that the applied mechanical strain is within the range of changes in blood pressure. However, the formation of clusters of myocytes after 24 hours stretch (fig. 2 A,B) may result in a decrease in the percentage elongation experienced by the cells, due to a diminished number of cellular adhesion sites interacting with collagen I at the bottom of the culture plate.

Reports in which the Flexcell apparatus was used to apply cyclic mechanical stretch to neonatal cardiomyocytes have measured, for instance, alterations in gene expression or cytokine production (Cadre et al., 1998; Yokoyama et al., 1999). Only Pimentel et al. (Pimentel et al., 2001) reported reactive oxygen species-mediated

apoptosis in 25% stretched, serum-deprived myocytes and Hirakawa et al. (Hirakawa et al., 1999) described apoptosis in dystrophin-deficient cardiac myocytes after 12 and 24 hours stretch in the Flexcell apparatus, but found no apoptotic cells in stretched and non-stretched (12 and 24 h) cardiomyocytes isolated from normal, dystrophin-proficient, neonatal hearts.

Secondly, we questioned whether the time course of the apoptotic process was chosen correctly. Suzuki et al. (Suzuki et al., 2001) have studied the time course of H₂O₂-induced apoptosis in adult rat ventricular cardiac myocytes. Their investigations indicate a rise in caspase-3 activity 4 hours after the start of treatment and occurrence of DNA fragmentation after 9 hours, completing the apoptotic process in about 14 hours. Yue et al. (Yue et al., 1998) described similar data for staurosporine-induced apoptosis in cultured neonatal rat myocytes. In their study, proteolytic activity was observed 4-8 hours after staurosporine addition, with a peak in the number of apoptotic cells 16 hours after stimulation (Yue et al., 1998). Both studies (Suzuki et al., 2001; Yue et al., 1998) are in accordance with our own staurosporine data. If mechanical stretch would induce apoptosis in our system it should have been visible in an experimental time course of up to 48 hours.

Thirdly, temporal changes in the start of the apoptotic process in combination with an overall low percentage of cell death can further underestimate the occurrence of apoptosis and make it nearly impossible to detect apoptosis above normal, healthy background levels in individuals. The percentage of apoptotic cells reported in in vitro studies and extrapolated to in vivo physiological events covers a wide range of (often unrealistic) data: 0.002% - 35% (Elsasser et al., 2000). Apoptotic rates of more than 1% would imply an almost complete loss of myocytes in 1 year.

The apoptotic process is characterized by a number of transient sequential events of which mitochondrial dysfunction, activation of caspases, and DNA fragmentation are most frequently studied. As a consequence, the time course to detect a specific marker is also transient and limited to a specific phase in the apoptotic process. Finally, apoptotic blebs and cell debris become undetectable when they are engulfed by neighbouring cells (Takemura et al., 2001).

Fourthly, the heart is exposed to a combination of various mechanical forces. Except from the normal contractions, the three-dimensional deformation of the heart tissue in vivo is dependent on wall stress, degree of hypertrophy, fibrosis, infarct area, etc.

We bypassed these effects by culturing the cardiac cells on collagen-coated plates. Cells are interconnected via gap junctions, to maintain intercellular

communication, and are coupled to the extracellular matrix via integrins that probably act as mechanotransducers (Ross and Borg, 2001). These physical connections initiate bi-directional signaling between cells that could be crucial for the ultimate outcome of a stimulus, survival or apoptosis. The observation by Simpson et al. (Simpson et al., 1999) that besides the degree of stretch also the direction of stretch is important in intracellular signaling, marks the complexity of the molecular pathways: stretch applied in parallel with the myofibrils results in other intracellular signals than stretch across the short axis of the myofilaments.

The tensegrity model, as postulated by the group of Ingber, implies that changes in cell shape as a result of mechanical stress directly influences many cell functions through changes in the cytoskeletal network that directly connects the nucleus to the cell surface (Wang et al., 2001). These reports (Ross and Borg, 2001; Simpson et al., 1999; Wang et al., 2001) prove that the interaction of the various signaling mechanisms and the pro- and anti-apoptotic actions of a stimulus are very complex and may depend on the cellular environment and intercellular communication.

Fifthly, recent publications suggest that apoptotic stimuli in the dysfunctional heart can also be derived from external factors. For instance, neutrophils infiltrating the ischemic myocardium express $\alpha 4$ -integrins that bind to myocytes and induce free radical production via the NADPH oxidase system (Poon et al., 2001). Another route of injury to the heart was presented by Scarabelli et al. (Scarabelli et al., 2001). Apoptosis of endothelial cells would be the first event in a time-dependent process following ischemia/reperfusion. Soluble factors, released from the apoptotic endothelial cells, would mediate apoptosis in myocytes. Also, pericardial fluid from patients with ischemic heart disease contains substances that induce apoptosis in cultured myocytes (Iwakura et al., 2001). Other external factors that modify the apoptotic response include hormones, plasma peptides and neurotransmitters (Hasegawa et al., 2001).

Thus, a good model for simulating pathophysiological situations should try to reconstitute the three-dimensional structure of the heart, as for instance in the engineered heart tissue model published by Fink et al. (Fink et al., 2000). Attention should also be paid to balanced culture conditions, as the presence or absence of survival factors influences the experimental results.

In conclusion, we could not find a direct link between mechanical stretch and apoptosis in an in vitro model system of cultured myocytes and fibroblasts. We recognize that in the working myocardium, molecular signaling cascades are much more complex than those in monolayer cultures. Particularly the effect of chronically reduced cardiac function appears to be difficult to reproduce in a cell

experiment. Our next goal is to elucidate the stretch-induced changes in gene expression and protein synthesis with the use of DNA microarrays. It will enable us to unravel and correlate several molecular signaling cascades at the same time in a simple in vitro model system, that may help in elucidating the events occurring in response to stretch.

Acknowledgements

The authors wish to thank mr. P. de Jong of the Animal Care Facilities, ms. E.J.M. van der Valk and ms. W.H. Bax for technical assistance.

This study was supported by the Dutch Heart Foundation # 97.176.

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3

Integrin stimulation induces calcium signaling in rat cardiomyocytes by a NO-dependent mechanism.

Chapter 3

Integrin stimulation induces calcium signaling in rat cardiomyocytes by a NO-dependent mechanism.

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Pflügers Archiv-European Journal of Physiology

In press

Abstract

The myocardial stretch-induced increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is considered to be caused by integrin stimulation. Myocardial stretch is also associated with increased nitric oxide (NO) formation. Our hypothesis was that NO is implicated in calcium signalling upon integrin stimulation.

Integrins of neonatal rat cardiomyocytes were stimulated with a pentapeptide containing the Arg-Gly-Asp (RGD) sequence. $[\text{Ca}^{2+}]_i$ was measured with Fura2, $[\text{NO}]_i$ was measured with DAF2, and activation of signaling intermediates was monitored with immunofluorescent techniques.

Integrin stimulation induced a rise in $[\text{NO}]_i$ and an increase in $[\text{Ca}^{2+}]_i$. The latter was inhibited by RyR2 blockers and by L-NMMA, an inhibitor of NOS, but resistant to GdCl_3 , diltiazem and Wortmannin. Consequently, integrin-induced $[\text{Ca}^{2+}]_i$ release appears to be independent of influx of extracellular Ca^{2+} ions and PI3K activity. In addition, integrin stimulation induced phosphorylation of FAK.

Our results provide evidence for an integrin-induced Ca^{2+} release from RyR2 which is mediated by NO formation via FAK-induced NOS activation.

Key words

Cardiomyocytes, integrins, NO, calcium signaling, FAK, NOS, RyR2

Introduction

Myocardial overloading, such as occurs during pressure overload or volume overload of the heart, initiates the development of myocardial hypertrophy. The biological mechanisms by which overload is sensed by the myocardium include Ca^{2+} influx through stretch-activated channels (Garnier et al., 1994; Tatsukawa et al., 1997), autocrine / paracrine mediators that are released by stretch and activate hormone receptors (Alvarez et al., 1999; Sadoshima et al., 1993), and integrin stimulation (Chicurel et al., 1998; Ingber, 2002; Shyy and Chien, 1997).

Integrin ligands, such as Arg-Gly-Asp (RGD) sequences present in fibronectin and other extracellular matrix (ECM) proteins (Ruoslahti and Pierschbacher, 1986),

have been shown to modulate cellular calcium homeostasis. In arteriolar smooth muscle cells (VSMCs), Madin-Darby canine kidney epithelial (MDCK) cells, and cardiomyocytes, integrin ligands produced either an increase of the L-type Ca^{2+} current ($I_{\text{Ca,L}}$) (Sjaastad et al., 1996; Waitkus-Edwards et al., 2002; Wu et al., 1998) or an increased Ca^{2+} release from the sarcoplasmic reticulum (SR) (Chan et al., 2001; Somogyi et al., 1994).

Myocardial stretch is also associated with increased nitric oxide (NO) formation in the tissue (Pinsky et al., 1997; Prendergast et al., 1997; Vila Petroff et al., 2001), as is the force-frequency response (Khan et al., 2003). NO modulates the characteristics of Ca^{2+} entry channels in the sarcolemma and Ca^{2+} release channels (RyR2) of the SR. $I_{\text{Ca,L}}$ was modified by SIN-1, a NO donor, in ferret ventricular myocytes (Campbell et al., 1996) and in frog ventricular cells (Méry et al., 1993), due to processes like S-nitrosylation of protein-thiols and NO-induced activation of cGMP-dependent protein kinases. The β -adrenergic stimulation of $I_{\text{Ca,L}}$ was augmented by SNAP, a S-nitrosothiol (Abi-Gerges et al., 2002), and $I_{\text{Ca,L}}$ of L-type Ca^{2+} channels expressed in human embryonic kidney (HEK293) cells was inhibited by several S-nitrosothiols (Hu et al., 1997). The Ca^{2+} release from RyR2 was activated by NO, as demonstrated in SR vesicles from canine ventricular tissue (Stoyanovsky et al., 1997), in HEK293 cells expressing the skeletal RyR (Sun et al., 2001), in RyR2 purified from canine hearts (Xu et al., 1998), and in rat cardiac myocytes (Vila Petroff et al., 2001).

NOS1 knockout mice had suppressed frequency-dependent sarcomere shortening and Ca^{2+} transients, compared to wild-type mice (Khan et al., 2003), compatible with the concept that NO regulates Ca^{2+} release from RyR2, presumably by S-nitrosylation (Martinez and Lamas, 2004; Stamler et al., 2001).

Using cultured rat cardiomyocytes we investigated whether the stretch-induced increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is caused by (i) integrin stimulation, (ii) stimulation of nitric oxide synthase (NOS), or (iii) both, if integrin stimulation would lead to increased NO formation. To that purpose we measured $[\text{NO}]_i$ and $[\text{Ca}^{2+}]_i$ in neonatal rat ventricular cardiomyocytes grown on glass coverslips coated with collagen, and stimulated integrins with a pentapeptide containing the RGD sequence. We found evidence for an integrin-induced Ca^{2+} release from RyR2 which is mediated by NO formation via focal adhesion kinase (FAK)-induced NOS activation.

Methods

Cell cultures

Neonatal (2-days old) rat ventricular cardiomyocytes were prepared as described before (van der Wees et al., 2003). Briefly, cells were dissociated with collagenase (Worthington Biochemical Corporation), preplated in plastic dishes (Falcon primaria, Becton-Dickinson) to allow preferential attachment of non-cardiomyocytes. One hour later non-adherent cells were collected and transferred to petri dishes (Ø 35 mm) each containing a glass coverslip (Ø 25 mm) that was coated with rat tail collagen (Sigma; 20 µg/mL). Culture medium consisted of Ham F-10 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 10% heat-inactivated horse serum (HS, Gibco), 100 U/mL penicillin (Yamanouchi) and 0.1 g/L streptomycin (Radiumpharma-Fysiopharma). Eight hours after seeding, culture medium was replaced by a medium containing Ham-F10 and DMEM (1:1 v/v), 5% HS and antibiotics. The cultures were grown in a humidified incubator at 37°C and 5% CO₂.

Fourty-eight hours after seeding, the medium was replaced by a medium containing Ham-F10 and DMEM (1:1 v/v), 2.5% HS and antibiotics.

The experiments had the approval of the Animal Experiments Committee of the institution, installed by Leiden University, according to Dutch law.

Experimental protocol

Seventy-two h after seeding cardiomyocytes onto the collagen-coated glass coverslips the cultures were loaded with 2 µM Fura2-AM (Molecular Probes) in HEPES buffered salt solution (HBSS), containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄, 2.5 mM CaCl₂, 10 mM NaHCO₃, 20 mM HEPES, pH 7.4, supplemented with 5.5 mM glucose and 2.5% FBS, for 45 min.

Next, the glass slide was transferred to a open-centered teflon dish and secured with an aluminum ring (Ince et al., 1985) filled with 1 mL of HBSS supplemented with 5.5 mM glucose, 2.5 mM probenecid (Sigma) and 2.5% FBS at 37°C. During the first hour cells were preincubated with or without inhibitors, such as 10 µM Wortmannin (inhibitor of phosphatidylinositol-3 kinase), 100 µM diltiazem (blocker of the L-type Ca²⁺ channel), 10 µM procaine and 10 µM Ruthenium Red (blockers of Ca²⁺ release from the SR), 100 µM ryanodine (blocker of Ca²⁺ release from the SR), 100 µM gadolinium chloride (blocker of the stretch-activated cation

channels), 50 μ M BAPTA-AM (Molecular Probes) to chelate intracellular Ca^{2+} ions, or with substances that either supply NO, such as the NO-donors sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) or block nitric oxide synthase (NOS), such as L-NMMA. Thereafter, the dish was fitted in a thermostated device (Ince et al., 1983) mounted on the stage of an inverted microscope (Leitz Diavert) and the effects of added substances, such as the pentapeptides Gly-Arg-Gly-Asp-Ser (containing the RGD sequence) and Ser-Asp-Gly-Arg-Gly (no RGD sequence; both from Sigma) and sodium-nitroprusside (SNP, an NO-donor), on $[\text{Ca}^{2+}]_i$ were measured.

Digital imaging fluorescence microscopy of Fura-2 loaded cardiomyocytes

This set-up has been described before (Atsma et al., 1995; Ruwhof et al., 2001). The microscope (Leitz Diavert, Wetzlar) had an excitation wavelength of 380 nm. Emission fluorescence of the cells under study was led through a 490 nm high-pass filter and was imaged by a high-sensitivity video camera (C2400-08, Hamamatsu). The video signal was digitized by a personal computer equipped with dedicated image processing software (TIM®, Difa) for image processing and statistical analysis. Background fluorescence was subtracted from the 380 nm image. Pixels that did not represent part of a cell (so called “extracellular pixels”) were set to zero using a template routine. Statistical routines excluded pixel values of zero to obtain statistical information, such as median values, of “cellular pixels” only.

NO imaging by digital imaging fluorescence microscopy

Cardiomyocytes grown on glass coverslips were washed twice with HBSS, and subsequently loaded with 10 μ M 4,5-diaminofluorescein diacetate (DAF2-DA, Alexa Biochemicals) (Nakatsubo et al., 1998) supplemented with 5 mM glucose, for 30 min at 22°C. After rinsing three times with HBSS, the glass coverslip was inserted in the teflon holder, 1 mL of prewarmed HBSS and 5 mM glucose was added, and the dish was mounted on the stage of the fluorescence microscope (Zeiss Axiovert 200M) controlled by computer equipped with imaging software (OpenLab, Improvion). The temperature of the medium was maintained at 37°C. Excitation fluorescence was 488 nm and emission fluorescence was led through a 515 nm high-pass filter. In order to minimise photo damage and/or bleaching of

DAF-2, fluorescence was measured discontinuously and using a 10% transmission grey filter in the excitation light path.

Immunofluorescence

Cardiomyocyte cultures were rinsed three times with ice-cold PBS + 1% FBS, incubated with ice-cold PBS + 1% formalin for 30 min, washed twice with ice-cold PBS + 1% FBS, incubated with ice-cold PBS + 0.1% Triton-X100 for 10 min, washed twice with ice-cold PBS + 1% FBS, and incubated overnight with first antibody (200x diluted in PBS) at 4°C. After washing twice with PBS + 1% FBS, the cells were incubated with secondary FITC-conjugated antibody (500x diluted) for 30 min. Following two wash steps with PBS + 1% FBS, the cultures were incubated with 10 µg/mL Hoechst 33342 (Molecular Probes) for 10 min. After washing twice with PBS + 1% FBS, cells were mounted on microscope slides using Vectashield (Vector). Rabbit antibody raised against phosphorylated FAK (Tyr-397) was from Santa Cruz (SC-11765-R). Goat anti-rabbit secondary FITC-conjugated antibody was from Sigma (F9887). Immunofluorescence images were produced using a fluorescence microscope (Nikon Eclipse) equipped with 100x oil-immersion objective and a digital camera (Nikon DXM1200). Images were analysed quantitatively using Image-Pro Plus software (Media Cybernetics).

Statistics

Results are expressed as mean ± SD. Statistical analysis was performed by Student t-test and by analysis of variance wherever appropriate. Differences were regarded as statistically significant when $p < 0.05$.

Results

Effects of RGD on $[Ca^{2+}]_i$

The pentapeptide Gly-Arg-Gly-Asp-Ser (RGD-peptide) caused an increase in $[Ca^{2+}]_i$ that was dose-dependent (figure 1A). Figure 1A also shows that the calcium-increasing effect of RGD-peptide developed slowly, with only slight effect in the first 5 min, and rising only if the RGD concentration was beyond a "threshold concentration" of 100 µg/mL.

Interventions that (I) inhibited PI3K activity (Wortmannin, 1 μ M), (II) blocked L-type Ca^{2+} channel conductance (diltiazem, 100 μ M), (III) and blocked stretch-activated cation channel conductance (gadolinium chloride, 100 μ M) had no effect on the RGD-peptide induced increase of $[\text{Ca}^{2+}]_i$ (figure 1B). However, inhibition of the ryanodine-sensitive Ca^{2+} release channel of the SR by a combination of procaine and Ruthenium Red (P+RR, both 10 μ M) prevented the RGD-peptide induced increase of $[\text{Ca}^{2+}]_i$ (figure 1B). Results with ryanodine (100 μ M) were completely similar to those with the combination of procaine and Ruthenium Red (both 10 μ M) (results not shown). Even in the absence of extracellular Ca^{2+} ions, RGD-peptide (500 μ g/mL) still produced an increase of $[\text{Ca}^{2+}]_i$ (results not shown). The effect of RGD-peptide appeared to be dependent upon the presence of NO. Sodium nitroprusside (SNP 500 μ g/mL) increased the Ca^{2+} rising effect of RGD, whereas a nitric oxide synthase inhibitor (L-NMMA, 50 μ M) abolished it (figure 1B).

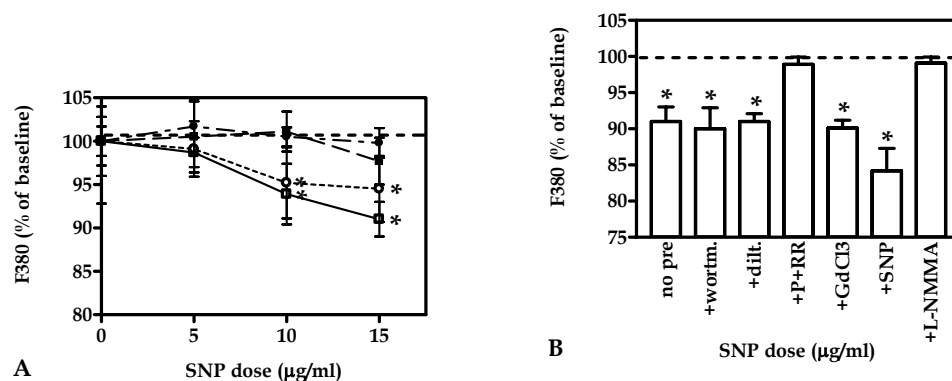


Figure 1 A. Effect of three doses of RGD-peptide on Fura2 fluorescence at 380 nm, representing an inverse measure of $[\text{Ca}^{2+}]_i$, expressed as a percentage of baseline value. The doses used were 0 ($\circ$ — $\circ$), 100 ($\bullet$ — $\bullet$), 200 ($\blacktriangledown$ — $\blacktriangledown$) and 300 ($\blacksquare$ — $\blacksquare$) μ g/mL. Indicated are mean values $\pm$ SD. * $p < 0.05$ compared to 0 μ g/mL RGD at similar time point. B. Effect of 15-min preincubation with Wortmannin (1 μ M), diltiazem (100 μ M), the combination of procaine and Ruthenium Red (P+RR, both 10 μ M), gadolinium chloride (100 μ M), sodium nitroprusside (SNP, 500 μ g/mL) and L-NMMA (50 μ M) on RGD-peptide (300 μ g/mL)-induced decrease of Fura2 fluorescence at 380 nm, representing an inverse measure of $[\text{Ca}^{2+}]_i$, expressed as a percentage of baseline value. Indicated are mean values $\pm$ SD. * $p < 0.05$ compared to baseline value.

Effects of NO on $[\text{Ca}^{2+}]_i$

Sodium nitroprusside (SNP) caused an increase in $[\text{Ca}^{2+}]_i$ that was dose-dependent (figure 2A). Interventions that blocked L-type Ca^{2+} channels (diltiazem, 100 μ M) and the ryanodine-sensitive Ca^{2+} release channel of the SR (by a combination of

procaine and Ruthenium Red, both 10 μ M) had a substantial, but not complete inhibiting effect on the SNP-induced increase of $[Ca^{2+}]_i$ (figure 2B). If the cardiomyocytes were loaded with BAPTA (10 μ M), addition of SNP did not cause a Ca^{2+} rise.

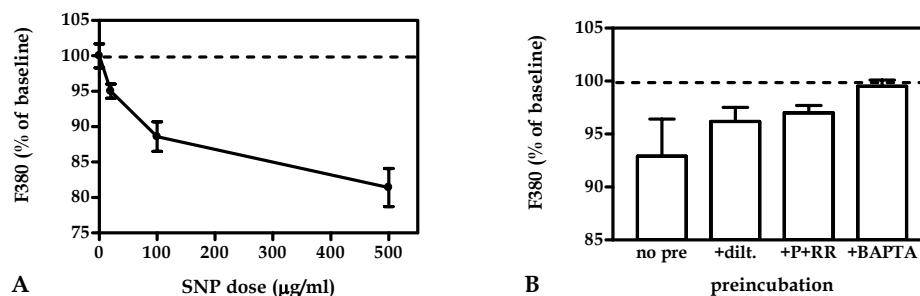


Figure 2 A. Concentration-dependent effect of sodium nitroprusside (SNP) on Fura2 fluorescence at 380 nm, representing an inverse measure of $[Ca^{2+}]_i$, expressed as a percentage of baseline value. Incubation time was 5 min. Indicated are mean values $\pm$ SD. **B.** Effect of diltiazem (100 μ M), the combination of procaine and Ruthenium Red (P+RR, both 10 μ M), and BAPTA-AM (10 μ M) on sodium nitroprusside (SNP, 100 μ g/mL)-induced decrease of Fura2 fluorescence at 380 nm, representing an inverse measure of $[Ca^{2+}]_i$. Incubation time was 5 min. Indicated are mean values $\pm$ SD. * $p < 0.05$ compared to baseline value.

Effects of RGD on $[NO]_i$

Addition of RGD to the cultures induced a time-dependent and dose-dependent increase of $[NO]_i$, whereas RGE was without such effects (figure 3).

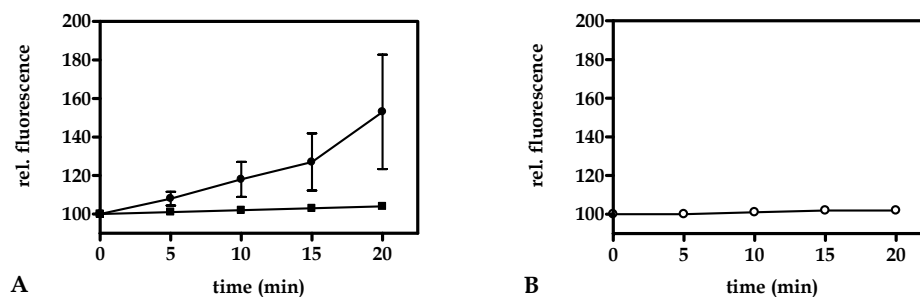


Figure 3 A. The effect of two concentrations (100 and 300 μ g/mL) of RGD on fluorescence of the NO indicator, DAF-2, expressed as intensity level relative to baseline fluorescence. Indicated are mean values $\pm$ SEM ($n=3$ per data point). **B.** The effect of several concentrations (50 – 300 μ g/mL) of RGE on fluorescence of the NO indicator, DAF-2, expressed as intensity level relative to baseline fluorescence. Indicated are mean values $\pm$ SEM ($n=4$ per data point).

The RGD-induced NO rise was observed only if the RGD concentration was beyond a "threshold concentration" of 100 $\mu\text{g/mL}$. In the presence of RGD, the rise of $[\text{NO}]_i$ exhibited a slow onset in the first 10 min, and accelerated thereafter. In contrast, the addition of SNAP (5 or 10 $\mu\text{g/mL}$) or SNP (5, 10 or 30 $\mu\text{g/mL}$) resulted in a rapid increase in intracellular NO, being about 10% of baseline levels after the first 5 min at a dose of 5 $\mu\text{g/mL}$ SNAP or SNP.

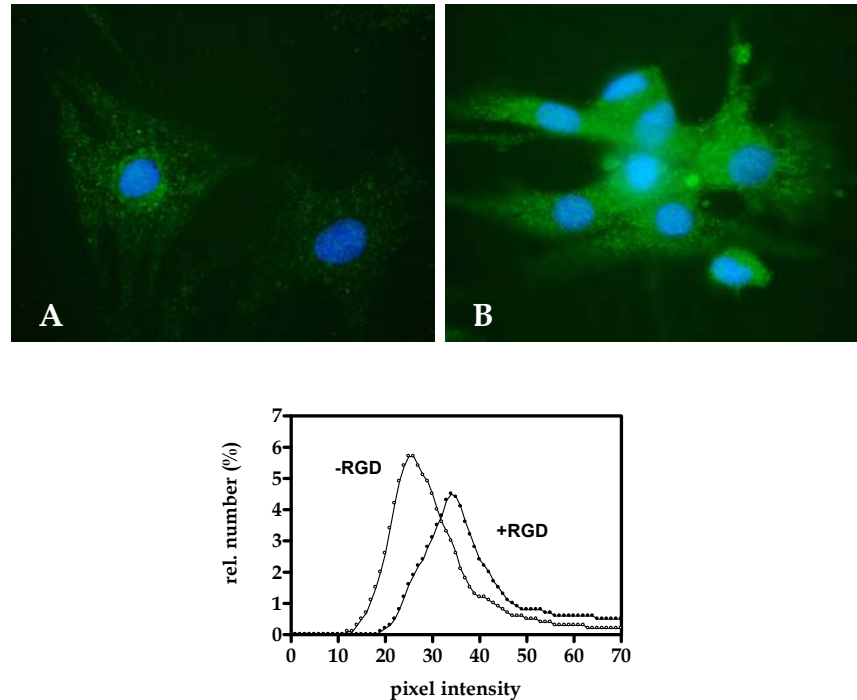


Figure 4. Immunofluorescent images of phosphorylated FAK (green) and nuclei (blue) in cardiomyocytes that were preincubated with RGD-peptide (100 $\mu\text{g/mL}$) for 1 hour and incubated with RGD-peptide (400 $\mu\text{g/mL}$) for 1 hour (panel B) or with PBS replacing RGD-peptide solution during preincubation and incubation (panel A). The graph shows the two pixel intensity distributions.

RGD-peptide and focal adhesion kinase (FAK) activation

Cardiomyocytes preincubated with RGD-peptide (100 $\mu\text{g/mL}$) for 1 hour and incubated with RGD-peptide (400 $\mu\text{g/mL}$) for 1 hour showed markedly increased intensity of phosphorylated FAK (figure 4), indicating activation of FAK by RGD-peptide in a way that appears similar to the effect of stretch (unpublished observations).

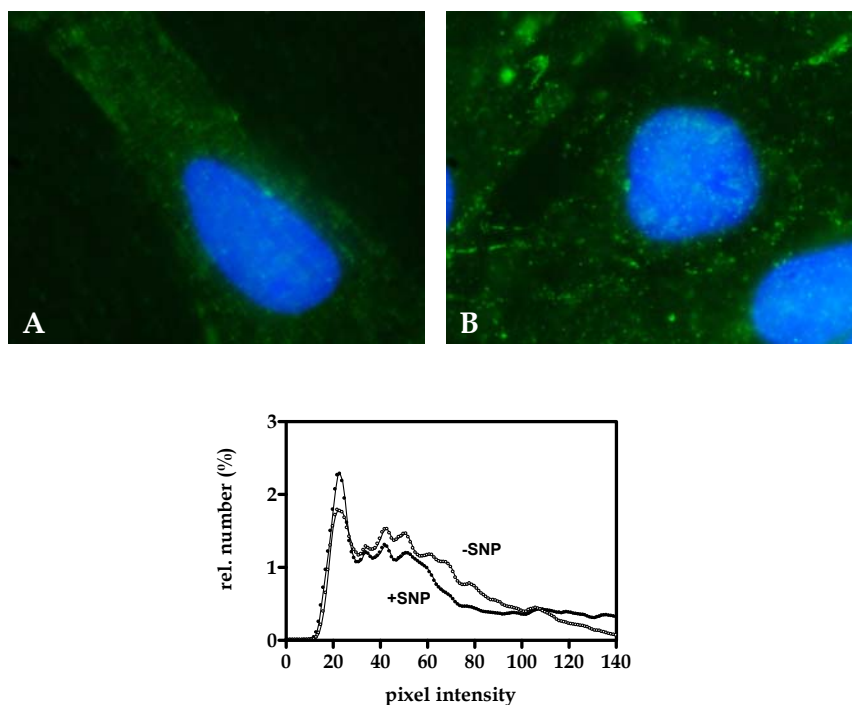


Figure 5. Immunofluorescent images of phosphorylated FAK (green) and nuclei (blue) in cardiomyocytes that were preincubated with RGE-peptide (100 µg/mL) (panel A) or with sodium nitroprusside (SNP, 100 µg/mL) (panel B) for 15 min. The graph shows the two pixel intensity distributions.

NO-induced FAK activation

Cardiomyocytes incubated with sodium nitroprusside (SNP, 100 µg/mL) for 15 min revealed a somewhat lower intensity of phosphorylated FAK than cardiomyocytes incubated with RGE-peptide (100 µg/mL) for 15 min (figure 5).

Discussion

The effect of RGD-peptide on cellular calcium signaling is dependent of dose, is inhibited by RyR2-blockers, and is independent of PI3K activity and L-type Ca^{2+} channel blockade. Given the slow response of RGD-peptide on increase of $[\text{Ca}^{2+}]_i$ and $[\text{NO}]_i$, integrin-induced stimulation is considered to be mediated by Ca^{2+} release from SR via stimulation of RyR2 conductance. The activation of RyR by NO

(Stoyanovsky et al., 1997; Sun et al., 2001; Vila Petroff et al., 2001; Xu et al., 1998) is illustrated by the potentiating effect of SNP and the blocking effect of L-NMMA on RGD-induced calcium signaling (see figure 1B).

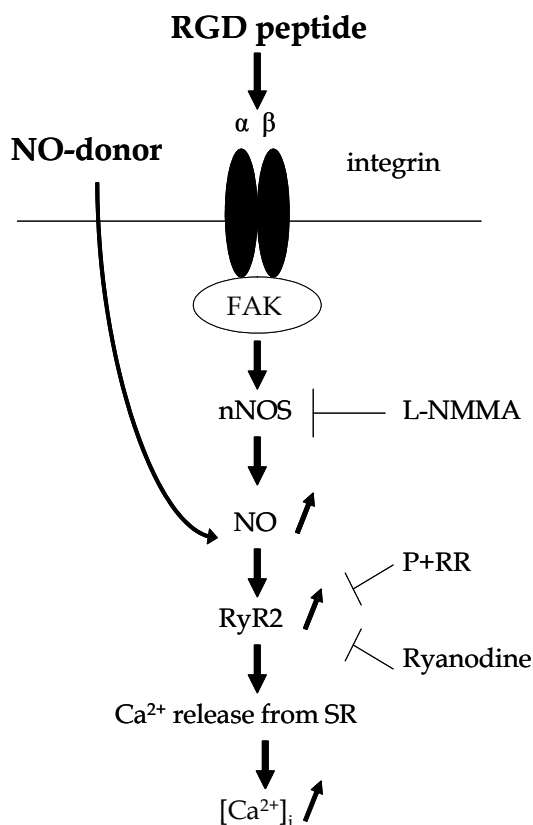


Figure 6. Schematic representation of signaling events involved in calcium signaling by extracellularly applied RGD-peptide and NO-donors.

RGD-peptide causes integrin-mediated FAK activation, but an effect on Akt activation seems unlikely, as an inhibitor of PI3K, Wortmannin, was unable to block RGD-induced calcium signaling.

NO-donors, like SNP, also induce an increase of [Ca²⁺]_i that is not completely blocked by RyR2-blockers, nor by L-type Ca²⁺ channel blockers (see figure 2B). So it appears that NO, either via protein kinase G (PKG) activation or by NO-induced nitrosylation of RyR2 and/or L-type Ca²⁺ channels, causes increased conductance

of one or both channel types. The effects of RGD-peptide and NO are additive, adding evidence to independent pathways that converge at the level of RyR2. Vila Petroff et al. (2001) have demonstrated that stretch of cardiomyocytes modulated the Ca^{2+} release process from RyR2, which finding they ascribed to PI3K-dependent phosphorylation of both Akt and NOS3. In the present study, integrin stimulation by exogenous RGD-peptide was independent of PI3K inhibition, suggesting no role of Akt phosphorylation in the process of RGD-induced rises of Ca^{2+} and NO. However, the interplay between RGD-peptide and NO is evident, as L-NMMA inhibits the calcium-increasing effect of RGD-peptide. One of the explanations of the latter finding is that the RGD-peptide has a calcium increasing effect via increased activity of NOS, leading to NO-induced Ca^{2+} release from RyR2. Whether integrin-mediated FAK phosphorylation is a prerequisite for RGD-peptide induced NOS activation, is not yet fully proven, but the finding that NO-donors lack a FAK activating effect (see figure 5) fits in this theory.

NO-donors do not appear to modulate calcium signaling via FAK. Direct effects of NO on sarcolemmal Ca^{2+} channels (Campbell et al., 1996; Hu et al., 1997; Méry et al., 1993) and SR Ca^{2+} channels (Stoyanovsky et al., 1997; Sun et al., 2001; Vila Petroff et al., 2001; Xu et al., 1998) have been proposed by others. Normally, high pacing rates increases contractility and lusitropy, but in NOS1-knockout mice these responses to rapid pacing were markedly attenuated (Khan et al., 2003). However, cardiomyocytes isolated from NOS3-knockout mice had preserved force-frequency response (Khan et al., 2003), illustrating the role of NOS1-dependent NO formation in calcium handling of the SR. Therefore, the effects of integrin stimulation on Ca^{2+} signalling in cardiomyocytes reported in the present study are probably mediated by NOS1.

Our results on calcium signaling by RGD-peptide and NO-donors have led to a schematic representation of signaling events (figure 6). The influence of integrin stimulation on NOS activity via phosphorylated FAK that is closely associated with the cytoplasmic region of the integrin dimer involves an important intracellular messenger function of NO in regulating Ca^{2+} efflux from RyR2.

The negative effects of NO-donors on the phosphorylation of FAK may be explained by direct effects of NO on protein S-nitrosylation (Martinez_Ruiz and Lamas, 2004; Stamler et al., 2001), but are probably consequences of NO-induced cGMP production and PKG activation. To verify the latter explanation, future experiments will use inhibitors of guanylate cyclase and PKG. Furthermore, the results of the present study may stimulate the development of interventions that

specifically address the messenger function of NO in order to modulate aberrant RyR2 function.

Acknowledgements

This study was supported by the Dutch Heart Foundation #97.176. We thank Salma Gurmany for assistance with NO imaging.

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4

Deficient global genome repair of UV-induced cyclobutane pyrimidine dimers in terminally differentiated myocytes and proliferating fibroblasts from the rat heart.

Chapter 4

Deficient global genome repair of UV-induced cyclobutane pyrimidine dimers in terminally differentiated myocytes and proliferating fibroblasts from the rat heart.

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Abstract

Nucleotide excision repair (NER) is the principal pathway for the removal of a wide range of DNA helix-distorting lesions. Two NER subpathways have been identified, i.e. global genome repair (GGR) and transcription-coupled repair (TCR). Little is known about the expression of NER pathways in differentiated cells. We assessed the repair of UV-induced cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4 PP) in terminally differentiated myocytes and proliferating fibroblasts isolated from the hearts of neonatal rats. Myocytes and fibroblasts were found to carry out efficient removal of 6-4 PP but display poor repair of CPD by GGR. Furthermore both cell types were found to carry out TCR of CPD, thus mimicking the repair phenotype of established rodent cell lines. The inefficient repair of CPD at the genome overall level occurs in the absence of massive apoptosis, but goes along with an undetectable level of transcription of the p48 gene, known to be mutated in xeroderma pigmentosum group E (XP-E) patients and recently proposed to be essential for repair of CPD in nonexpressed DNA. Taken together, the results suggest that primary non-dividing cardiac myocytes and proliferating fibroblasts from rat heart selectively remove CPD from the transcribed strand of transcriptionally active genes. GGR of CPD is poor due to the absence of p48 expression.

Keywords

terminally differentiated cardiac myocytes, proliferating cardiac fibroblasts, UV-induced photolesions, nucleotide excision repair, apoptosis, p48

Introduction

The mammalian heart contains several cell types among which the myocytes and the fibroblasts are most abundant. Myocytes are essential for contractile function whereas fibroblasts generate the extracellular matrix. It is known that the genome of any cardiac cell is the target of DNA damaging agents originating from multiple sources, both exogenous (due to radiation and chemicals) and endogenous (due to

the reactive products of cellular oxidative metabolism). To cope with the deleterious consequences of DNA lesions, cells are equipped with efficient defense mechanisms. Such mechanisms include the removal of DNA damage by DNA repair pathways, control of cell cycle progression and the elimination of damaged cells via apoptosis. Cardiac myocytes lose the ability to proliferate shortly after birth (Katzberg et al., 1977), and hence extensive apoptosis will deteriorate heart function via an uncompensated loss of myocytes, resulting in a loss of pump function of the whole heart and ultimately heart failure (Narula et al., 1996). Therefore repair of DNA damage is crucial for these cells to maintain proper function.

Nucleotide excision repair (NER) is a versatile repair pathway involved in the removal of a broad variety of structurally unrelated DNA lesions. NER removes bulky lesions that distort the DNA helix, including UV-induced cyclobutane pyrimidine dimers (CPD) and (6-4)-photoproducts (6-4 PP), bulky adducts induced by chemicals and several lesions produced by reactive oxygen species (ROS) (Lunec et al., 2002; Moller and Wallin, 1998). Two NER subpathways have been identified, i.e. global genome repair (GGR) and transcription coupled repair (TCR) (Friedberg et al., 1995). GGR removes DNA lesions throughout the genome, whereas TCR acts specifically on DNA lesions in the transcribed strand of active genes. The efficiency of repair of DNA lesions by TCR and GGR in mammalian cells depends on a number of factors, including species, cell type and type of DNA lesion. Most notably, repair efficiency of UV-induced CPD appears to differ distinctly between rodent and human cells (van Zeeland et al., 1981). Most rodent cells are deficient in repair of UV-induced CPD in bulk DNA and hence exhibit a defect in GGR of CPD. In contrast TCR of CPD in these cells appears to be very efficient resulting in selective repair of CPD in the transcribed strand of expressed genes. Human cells exhibit both GGR and TCR of CPD resulting in the preferential repair of CPD in the transcribed strand of expressed genes.

Efficient GGR of CPD in human cells requires a DNA damage binding activity (DDB) with a high affinity for damaged DNA, mediated by a complex of two protein subunits, p125 and p48, the latter playing a critical role in the nuclear localization of p125 (Shiyanov et al., 1999). Binding activity and p48 expression are absent in a subset of xeroderma pigmentosum group E (XP-E) patients (Nichols et al., 1996), in wild type hamster cell lines (Radic, V et al., 1998) and in several primary tissues from hamsters and mice (Tang et al., 2000); these cells are all characterized by a deficiency in GGR of CPD. Injection of purified human DDB protein into XP-E cells stimulated GGR to levels observed in normal human cells

(Keeney et al., 1994) and transfection of hamster cells with human p48 gene enhanced GGR of CPD (Tang et al., 2000).

Despite the knowledge of NER in human and rodent fibroblasts and established cell lines, little is known about NER and the expression of the p48 gene in terminally differentiated cells. Using repair-competent cell extracts from different tissues of the rat Gospodinov et al (Gospodinov et al., 2003) demonstrated a correlation between NER efficiency and the proliferative status of the tissue, with rapidly proliferating tissue showing higher NER capacity than slowly proliferating tissue such as brain and heart. In the present study we addressed the question of NER efficiency and cellular differentiation by focusing on expression of NER and transcription of the p48 gene in terminally differentiated myocytes and proliferating fibroblasts isolated from the hearts of neonatal rats employing UV-light as a model genotoxic agent. The selective removal of CPD from the transcribed strand of transcriptionally active genes demonstrate that proliferation status has no impact on repair phenotype: both terminally differentiated myocytes and proliferating fibroblasts from the rat heart exhibit efficient TCR and poor GGR. Moreover the poor GGR of CPD parallels the absence of p48 expression.

Materials and methods

Cell cultures

Preparation of neonatal rat cardiac myocytes and fibroblasts:

Primary cultures of ventricular myocytes and fibroblasts were prepared from two-day-old male Wistar rats as described below. Hearts were excised from rats anesthetized with diethyl ether and transferred to solution A (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 5.6 mM D-glucose, 20 mM HEPES and 0.02% Phenol red, pH 7.4), after which ventricles were separated and minced into small fragments. Tissue fragments were further dissociated by incubating them twice with an enzyme solution containing 450 U collagenase type I (Worthington Biochemical Corporation) and 14 U DNase per ml solution A in a shaking water bath at 37°C for 30 min. Pooled cell suspensions were cooled on ice, pelleted by centrifugation (15 min, 1000g), resuspended in growth medium consisting of Ham's F10 supplemented with 10% fetal bovine serum (FBS), 10%

horse serum (HS), 100 U/ml penicillin and 100 µg/ml streptomycin, and seeded in primaria-coated culture dishes (Falcon, Beckton Dickinson).

Myocytes were separated from non-myocytes (mainly fibroblasts) by using a selective adhesion technique: the rate of attachment of non-myocytes to the culture-dishes is higher than that of the myocytes. After 60 min the medium was transferred to another dish providing cultures enriched in cardiac myocytes. Myocytes were allowed to attach for 4h after which growth medium was replaced by culture medium consisting of DMEM/Ham's F10 (1:1) supplemented with 5% HS, 100 U/ml penicillin and 100 µg/ml streptomycin. After incubation for 48h at 37°C in a humidified incubator with 5% CO₂ atmosphere, the medium was replaced with DMEM/Ham's F10 (1:1) containing 2.5% HS, 100 U/ml penicillin and 100 µg/ml streptomycin, and cells were incubated for another 24h.

Adherent cells, mainly fibroblasts, obtained during the preplating procedure described above were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Two days later the medium was replaced by DMEM supplemented with 2.5 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and cells were incubated for another 24h.

Using this method we routinely obtained confluent cultures containing either spontaneously contracting cardiac myocytes or nearly pure cardiac fibroblasts. The use of animals for this study has been approved by the Institutional Committee on Animal Experiments, as required by Dutch law.

Cell cultures used as control:

Two human fibroblast cultures obtained from human fore skin (VH16 and VH25) were used as positive control in this study. These cells were cultured in Ham's F10 medium without hypoxanthine and thymidine and supplemented with 15% fetal calf serum and antibiotics, in a 5% CO₂ atmosphere.

UV irradiation

Myocytes and fibroblasts were irradiated with ultraviolet light 72h after isolation from the hearts of two-day-old neonatal rats. Prior to irradiation the culture medium was removed and the cells were rinsed with phosphate buffered saline (PBS). Subsequently the monolayer of cells, either myocytes or fibroblasts, was irradiated with UV-C (Philips T.U.V., predominantly 254 nm) at a dose rate of 1.25 J/m²/s.

Analysis of UV-induced cell death

Cell death was quantified by the release of cytoplasmic α -hydroxybutyrate dehydrogenase (α -HBDH) activity, an activity of the LDH-1 and -2 isoenzymes, from the myocyte cultures and lactate dehydrogenase (LDH) activity from the fibroblast cultures. Samples of incubation medium and cell homogenate (in PBS with 0.1% w/v Triton X-100) were assayed for α -HBDH and LDH activities using test kits (Roche). Percentual α -HBDH and LDH release reflects the percentage of cell death in the culture as was previously shown (van der Laarse et al., 1979).

Analysis of UV-induced apoptosis

DNA laddering:

To detect nucleosomal fragments generated by apoptosis, cells were UV-irradiated as described above and incubated for different time intervals in normal culture medium. Floating and adherent cells were collected separately, lysed and DNA was isolated. Five μ g DNA was treated with 1 μ l RNase (10,000 units/ml) for 1h at 37°C. The DNA was mixed with 1 volume of neutral loading buffer (40% sucrose, 0.05% bromophenol blue) and electrophoresis was performed in 2.0% agarose at 75 Volt for 2h in the presence of ethidium bromide to detect oligonucleosomal DNA fragmentation.

Caspase-3 activity:

Caspase-3 activity was measured by the cleavage of the fluorogenic DEVD-AMC conjugate according to Van de Water et al. (van de Water et al., 1999). Briefly, cells were collected, centrifuged and resuspended in lysis buffer (10 mM HEPES, 40 mM β -glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, pH 7.0), and subjected to three cycles of freezing and thawing. Cell lysates were centrifuged for 30 min at 13000 rpm and the protein concentration of the supernatants (cytosolic extracts) was measured with a BCA Protein Assay (Pierce). Cell lysate containing 40 μ g of protein was combined in duplicate with 25 μ M DEVD-AMC (Biomol), dissolved in reaction buffer (100 mM HEPES, pH 7.25, 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40, 10 mM DTT). DEVD-AMC hydrolysis was measured fluorometrically with a Victor 1420 multilabel counter (Wallac), using AMC fluorescence as a standard. The caspase activity was calculated from the linear

portion of the fluorescence versus the time curve and expressed as pmol AMC/min/mg protein.

Analysis of removal of CPD and 6-4 PP from the genome overall

Fluorescent labeling:

Cells, grown on coverslips, were washed twice with cold PBS at various time-points following UV-irradiation. Subsequently the cells were fixed on coverslips and permeabilized for 15 min by addition of PBS containing 1% formaldehyde and 0.5% Triton X-100 (Fluka) while maintaining them on ice. In order to visualize UV-induced photoproducts, cells were washed twice with PBS and treated with 0.2 M HCl for 15 min at 37°C. Following incubation with 5% bovine serum albumin (Sigma) in PBS for 60 min at room temperature cells were incubated overnight at 4°C with the appropriate primary antibody (mouse IgG monoclonal anti-CPD (TDM2) and anti-6-4 PP (64 M2) (1:2000 and 1:200, respectively), gifts from Dr. O. Nikaido, Kanazawa University, Kanazawa, Japan). Incubation with Cy3-conjugated mouse anti-rabbit IgG plus IgM (Jackson Laboratories, Westgrove, PA) was performed at room temperature for 30 min following three wash steps with PBS. After finishing the first labeling cells were again incubated with 5% bovine serum albumin (Sigma) in PBS for 60 min at room temperature after which they were incubated with the monoclonal mouse anti- α -actinin antibody (Sigma, clone EA-53) for 2h at RT. Thereafter, cells were incubated with FITC-conjugated rabbit anti-mouse IgG antibody (Sigma) for 30 min. After the last antibody labeling step, cells were mounted in mounting medium containing 4'-6'-diamidino-2-phenylindole (DAPI) as a DNA counter stain (Vector Laboratories, Burlingame, CA)

Fluorescence images were obtained with a Zeiss Axioplan 2 fluorescence microscope equipped with an AttoArc HBO 100W adjustable mercury arc lamp and fitted with appropriate filters for FITC, DAPI and Cy3. Digital images were captured with a cooled CCD camera (Hamamatsu C5935, Hamamatsu, Japan) and processed with the Metasystems ISIS software package (Metasystems, Altlussheim, Germany). To quantitate the fluorescence signals, 10 cells were randomly chosen from each sample, and the intensity of the fluorescent signal in the nucleus was measured and expressed in arbitrary units using the ISIS software.

Chapter four

Immuno dotblot assay:

High molecular weight DNA was applied to a nitrocellulose filter (0.1 μm) using a Dotblot apparatus (Schleicher and Schuell, minifold II). The filter was dried at 42°C, washed with PTB (PBS containing 0.05% Tween-20 and 0.1% BSA), blocked with sterilized skimmed milk containing 0.05% Tween-20 and incubated with mouse IgG monoclonal anti-CPD (1:2000, TDM2) or mouse IgG monoclonal anti-6-4 photoproduct (1:200) overnight at 4°C. After washing the filters were incubated with anti-mouse antibody conjugated with horseradish peroxidase, incubated with Supersignal west Dura solution (Pierce) and exposed to X-ray film. Repair proficient human VH25 skin fibroblasts were used as a positive control.

Analysis of RNA synthesis

For RNA synthesis measurements, myocytes and fibroblasts were prelabeled 30h after isolation with [^{14}C]-thymidine (0.01 $\mu\text{Ci}/\text{ml}$, 60 mCi/mmol) for 42h, washed twice with PBS and irradiated with different doses of UV (0, 5 and 10 J/m^2). At various times after UV-irradiation, the medium was replaced by fresh medium containing [^3H]-uridine (10 $\mu\text{Ci}/\text{ml}$, 43 Ci/mmol), and cells were incubated for 30 min at 37°C. Subsequently cells were washed twice with PBS and lysed in 10% sodium dodecyl sulfate (SDS) for 1h at room temperature (RT). RNA was precipitated with trichloroacetic acid (final concentration 10%) for 1h at 4°C and collected on Whatmann GF/C filters by vacuum filtration. The quantities of ^3H and ^{14}C on the filters were measured by liquid scintillation and the ratio of ^3H to ^{14}C incorporation was taken as a measure of RNA synthesis (van Hoffen et al., 1999).

Determination of CPD frequencies in specific DNA sequences

At different time intervals following UV-irradiation, cells were lysed in a buffer containing 150 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8.0, 0.5% SDS and 100 $\mu\text{g}/\text{ml}$ proteinase K either at 50°C for 2h or at 37°C overnight. High molecular weight DNA was purified by phenol and chloroform extractions, ethanol precipitated and resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was digested overnight with either EcoR1 at 37°C or Bcl1 at 50°C. Subsequently, DNA was purified again by phenol and chloroform extractions, ethanol precipitated and resuspended in TE.

DNA samples were subjected to T4 endonuclease V digestion and subsequently to alkaline gel electrophoresis, Southern blotting and hybridization as described

(Vreeswijk et al., 1994). Briefly, equivalent amounts of DNA, either treated with or without T4 endonuclease V, were loaded onto a 0.6% alkaline agarose gel. After electrophoresis DNA was transferred to Hybond N+ membranes (Amersham) by vacuum Southern blotting (Pharmacia-LKB Vacugene 2016). Filters were hybridized for 40h at 42°C in 3 ml of formamide containing hybridization solution, washed and scanned using an InstantImager™ Electronic Autoradiography System (Packard). The frequency of CPD was calculated from the relative band intensities of full size restriction fragments in the lanes containing DNA that was either treated or mock-treated with T4 endonuclease V (Ruven et al., 1994)[17]. After scanning, filters were exposed to Kodak X-Omat AR X-ray films at -70°C.

Northern blot analysis

Total cellular RNA was extracted using Trizol (Gibco) and analysed by Northern blotting. RNA samples (30 µg total RNA) were denatured by heating (15 min, 55°C) in sample buffer: 50 mM Na-acetate, 1 mM EDTA, 50% formamide, 7.5% formaldehyde in 20 mM 3-(morpholino)propanesulphonic acid (MOPS). After addition of loading buffer (25% Ficoll400, 0.4% Orange G in water) RNA was fractionated on a gel (1% agarose and 7.5% formaldehyde in MOPS). Following fractionation RNA was transferred to Hybond N+ membrane (Amersham) by capillary transfer in 20xSSC (3M NaCl, 0.3M Na-citrate) with 7.5 % formaldehyde for 16h, after which RNA was covalently attached by UV-cross-linking. Filters were hybridized for 16h at 42°C in 3 ml of formamide containing hybridization solution, washed and scanned using an InstantImager™ Electronic Autoradiography System (Packard).

Probes

A plasmid (pGAL), containing the entire Chinese hamster APRT gene, an HPRT cDNA fragment containing exon 6-9 or a fragment containing the 3' end of the c-mos gene, were used to prepare APRT, HPRT and c-mos-specific probes, respectively. Strand specific single-stranded probes were generated with [α -32P] ATP by a linear PCR using a single primer specifically recognizing one strand (Ruven et al., 1994). A probe specific for p48 was generated by linear PCR using a 160bp double stranded PCR product as a template and a single primer recognizing the transcribed strand (5'-TACGGAGGCTGTGGCCAGGA-3'). Linear PCR was

carried out in the presence of [α - ^{32} P] ATP. Double stranded probe specific for GAPDH was radioactively labeled with [α - ^{32} P] ATP by random primer extension.

Results

UV-induced apoptosis

Heart tissue of patients with heart failure typically contains apoptotic cells (Narula et al., 1999; Sabbah, 2000); however it is unknown whether this apoptotic cell death is caused by direct DNA damage or via other apoptosis-inducing factors. Experiments performed by Vreeswijk et al (Vreeswijk et al., 1998) indicated reduced DNA repair in apoptotic cells, already at the early onset of apoptosis. Therefore we examined whether cardiac cells are prone to apoptosis following DNA damage.

Enzyme release: Exposure of cardiac myocytes and fibroblasts to UV-irradiation (10 J/m²) did not result in massive irreversible cell injury. 24h post UV-irradiation the percentage of α -HBDH release from myocytes was $23.3 \pm 4.5\%$ versus $6.7 \pm 2.5\%$ in non-irradiated cells. Comparable results were seen 24h after UV-irradiation of fibroblasts: $28.3 \pm 2.5\%$ LDH release versus $10.8 \pm 2.3\%$ in control fibroblasts.

DNA laddering: Microscopic examinations of the monolayers of myocytes and fibroblasts revealed that a fraction of cells had detached from the culture dish 24h after UV-irradiation.

To determine whether cell detachment was caused by UV-induced apoptosis, DNA was isolated from adherent and floating cells immediately and 24h after irradiation. Neutral agarose gel analysis of DNA isolated from attached myocytes and fibroblasts, showed that DNA was still intact. DNA isolated from the detached cells (10-20% of the total cell population, comparable to the percentage of α -HBDH (myocytes) or LDH (fibroblasts) release) showed the nucleosomal laddering pattern characteristic for apoptosis, indicating that these cells underwent apoptosis after exposure to UV (Figure 1).

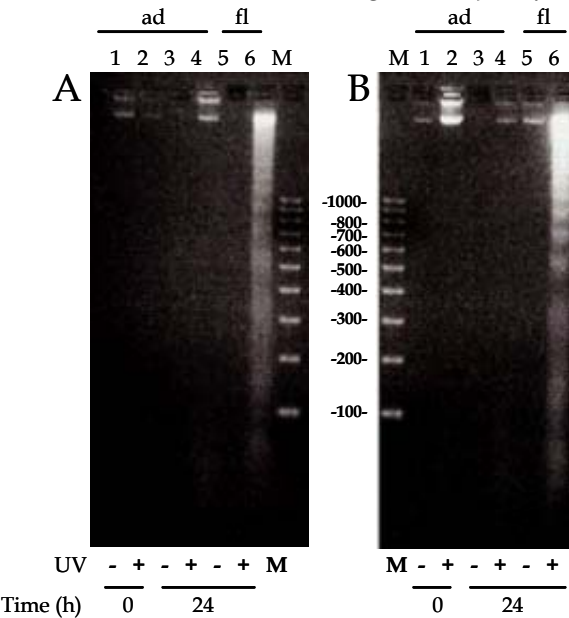


Figure 1. UV-induced nucleosomal degradation of DNA isolated from myocyte cultures (A) and fibroblast cultures (B) after irradiation with a UV-dose of 10 J/m². Genomic DNA was separated on a 2% neutral agarose gel and stained with ethidium bromide. Ad is DNA from adherent cells, fl is DNA from floating cells, plus is irradiated, - is unirradiated. The size marker (M) used is 100 bp molecular ruler (Biorad).

Caspase-3 activity:

Caspase-3 activity was measured in both adherent and floating cells of UV-irradiated and control myocyte and fibroblast cultures. In adherent myocytes, a 4-5 fold increase in caspase-3 activity could be observed 24h after irradiation, indicating that part of the cell population was irreversibly injured (Figure 2A). We note here that the level of caspase-3 activity in these irradiated cells is 10 times lower compared to caspase-3 activity measured in these cells under conditions that they display massive apoptosis induced by treatment with staurosporine (120 pmol/min/mg versus 1200 pmol/min/mg). The increase in caspase-3 activity in adherent fibroblasts measured 24h after irradiation was rather small (Figure 2B). In the culture medium, containing the floating cells and/or apoptotic bodies, a significant increase in caspase-3 activity could be observed. The total caspase-3 activity in the floating cells of irradiated myocyte cultures was 16.5 ± 1.9 pmol/min/petridish versus 0.2 ± 0.1 pmol/min/petridish in control cultures. In floating fibroblasts, the activity was 12.2 ± 3.9 pmol/min/petridish in UV-treated and 1.1 ± 0.4 pmol/min/petridish in non-treated cultures.

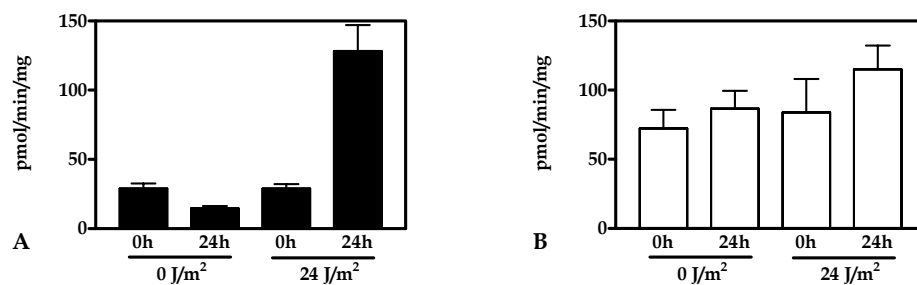


Figure 2. Caspase-3 activity measured in attached cells by the cleavage of the fluorogenic DEVD AMC conjugate. A: myocytes, B: fibroblasts. The activity was calculated from the linear portion of the fluorescence versus timeplot and expressed as pmol AMC/min/mg protein bars represent standard deviation.

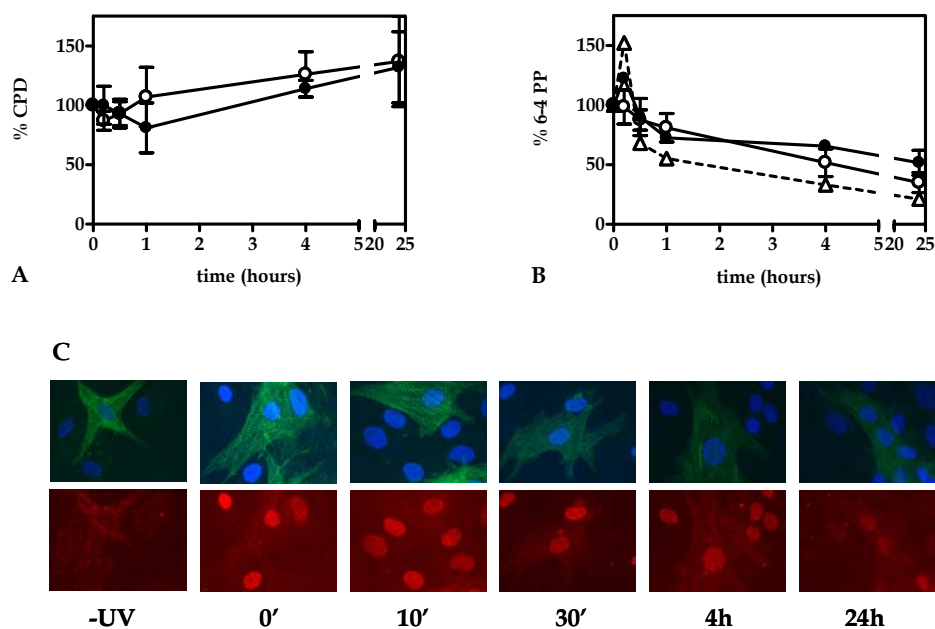


Figure 3. At various time points after global UV-irradiation (10 J/m²) cardiac cells were fixed and immunolabeled employing an antibody against CPD (A) or 6-4 PP (B). The fluorescent signal of 6-4 PP or CPD in the nuclei was quantified, normalized to the DAPI-stained nuclear area, averaged and set to 100% at 0h. To distinguish the cardiac myocytes from the cardiac fibroblasts cells were double stained with an antibody against the myocyte specific α -actinin. C double staining of 6-4 PP with α -actinin. Fibroblasts (open circle), myocytes (closed circle) VH16 (open triangle, dashed line), the error bars represent the SEM values of at least 30 nuclei.

Removal of CPD and 6-4 PP from the genome overall

Examination of the repair kinetics of the UV-induced 6-4 PP by immuno fluorescence revealed efficient removal of this photolesion at early time points after exposure to UV in human (VH16) as well as rat (cardiac) cells (Figure 3B and C). Since α -actinine is a myocyte specific antibody we were able to distinguish the cardiac myocyte from the cardiac fibroblast in mixed cell cultures and to measure repair by double staining of 6-4 PP and α -actinine, respectively. This approach revealed no difference in removal of 6-4 PP between the proliferating cardiac fibroblasts and the terminally differentiated cardiac myocytes (Figure 3B). Examination of the repair kinetics of CPD demonstrated no repair of this photolesion in myocytes as well as fibroblasts (Figure 3A).

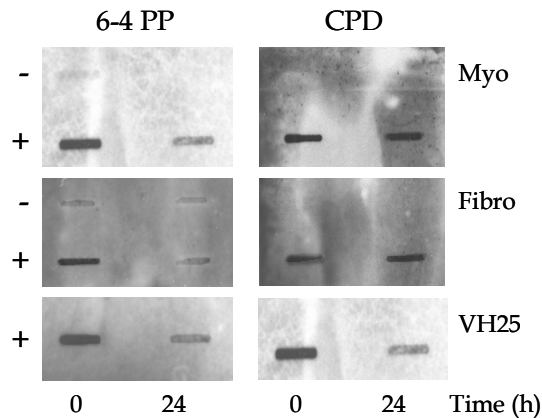


Figure 4. Analysis of the removal of CPD and 6-4 PP from the genome overall by immuno dotblot. Cells were UV-irradiated with a dose of 10 J/m². The presence of CPD and 6-4 PP was determined immediately after irradiation and 24h later. As a positive control repair proficient VH25 cells were used. + is irradiated, - is unirradiated.

Removal of cyclobutane pyrimidine dimers from defined genomic fragments

Repair of CPD was measured in cardiac myocytes and fibroblasts after irradiation with 10 J/m² UV-C by assessment of CPD frequencies in three genomic fragments, i.e. a 13.5 kb BclI fragment containing the entire sequence of the transcriptionally active autosomal APRT gene, a 17 kb BclI fragment of the transcriptionally active X-chromosomal HPRT gene and a 10 kb EcoRI fragment of the c-mos gene.

Since cells were derived from male rats, they contain two active APRT alleles and only one HPRT allele, which is also active.

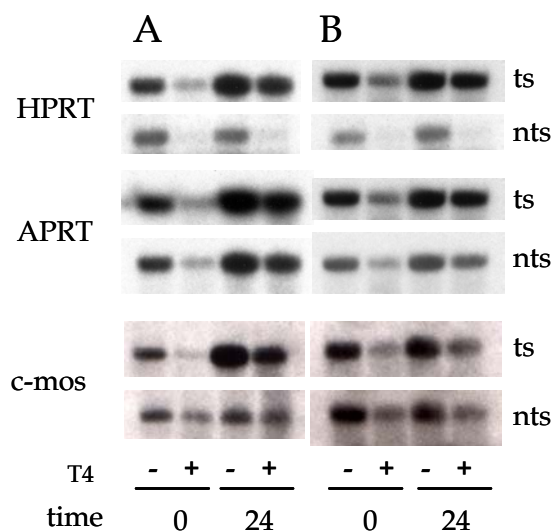


Figure 5. Representative autoradiograms of Southern blots showing removal of CPD after a UV-dose of 10 J/m² from the transcribed (ts) and non-transcribed (nts) of the active APRT and HPRT genes and the inactive c-mos gene in cardiac myocyte cultures (A) and fibroblast cultures (B). T4 is T4 endonuclease.

The presence of CPD in these fragments was measured using T4 endonuclease V, which specifically incises DNA at the site of a dimer, and quantitative Southern blot analysis. The repair of CPD is seen as the reappearance of the full-size restriction fragment in the T4 endonuclease V treated sample (Figure 5). Hybridization of filters with probes specific for the transcribed or non-transcribed strand of the HPRT gene, clearly demonstrated that in both cardiac myocytes and fibroblasts, CPD were only removed from the transcribed strand (56% and 73% respectively) and that repair of CPD in the non-transcribed strand was virtually absent 24h after UV-irradiation (Figure 5, table 1). In the APRT gene, however, CPD were removed from both strands in both cell types (Figure 5, table 1). Removal of CPD from the c-mos gene differed among the two cell types. In myocytes 52% of the CPD are removed from the transcribed strand after 24h, whereas virtually no CPD are removed from the non-transcribed strand (8%) in the same time span (Figure 5, table 1). Removal of CPD from both the transcribed and the non-transcribed strand in the c-mos gene in fibroblasts on the other hand did not significantly differ from that observed in the nts of the HPRT gene.

Table 1. Removal of CPD from the APRT, HPRT and *c-mos* gene in cardiac cells 24h after UV-irradiation (10 J/m²).

Gene fragment	probe	% removal of CPD 24h after irradiation $\pm$ se <i>c</i>	
		myocytes	fibroblasts
APRT 13.5-kb BclI	ts a	64 $\pm$ 6 (n = 3)	75 $\pm$ 2 (n = 3)
	nts b	40 $\pm$ 9 (n = 3)	67 $\pm$ 33 (n = 3)
HPRT 17-kb BclI	ts a	56 $\pm$ 7 (n = 3)	73 $\pm$ 5 (n = 2)
	nts b	3 $\pm$ 4 (n = 3)	15 $\pm$ 11 (n = 3)
<i>c-mos</i> 10-kb EcoRI	ts a	52 $\pm$ 8 (n = 2)	23 $\pm$ 7 (n = 2)
	nts b	8 $\pm$ 8 (n = 2)	17 $\pm$ 15 (n = 2)

a ts: transcribed strand*b nts*: non-transcribed strand*c se*: standard error

RNA synthesis recovery

Since ongoing RNA synthesis is essential for transcription-coupled repair, we determined whether cardiac myocyte and fibroblast cultures were able to recover from UV-inhibited RNA synthesis (Figure 6).

We used two different doses of UV and compared the incorporation of ³H-uridine in irradiated cells with the incorporation in non-irradiated cells at various times after UV. Upon exposure to 5 J/m², both myocytes and fibroblasts showed a marked inhibition of RNA synthesis: incorporation decreased to 50-60% compared to non-irradiated cells. Transcriptional activity completely restored to 100% in both fibroblasts and myocytes 24h after exposure to UV. Irradiation of both cell types at a higher dose of 10 J/m², which was also used for the analysis of CPD removal, inhibited RNA synthesis more strongly and led to limited restoration of transcription compared to treatment with 5 J/m².

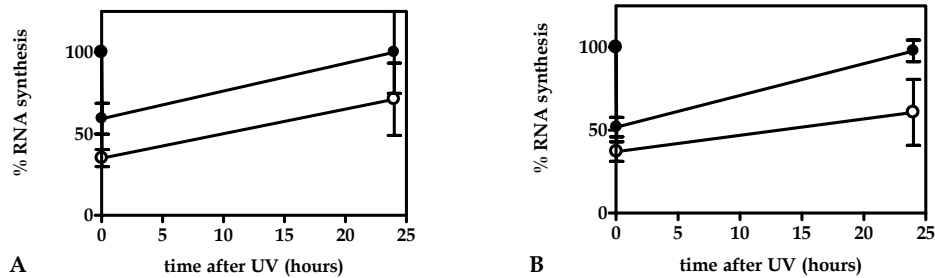


Figure 6. Recovery of UV-inhibited RNA synthesis following UV-irradiation. A: myocytes, 5 J/m² (closed circle), 10 J/m² (open circle); B: fibroblasts, 5 J/m² (closed circle), 10 J/m² (open circle). RNA synthesis was measured as the relative incorporation of ³H-uridine in UV-irradiated cells compared to unirradiated cells (100%). Bars represent standard deviation.

Expression of p48

The removal of CPD from the ts of the transcriptionally active HPRT gene is much more efficient when compared to removal of CPD from an inactive sequence in both cardiac myocytes and fibroblasts. This preferential repair is similar to that observed in rodent permanent cell lines. These permanent cell lines are deficient in GGR of CPD and lack the expression of p48, which is essential for GGR of CPD

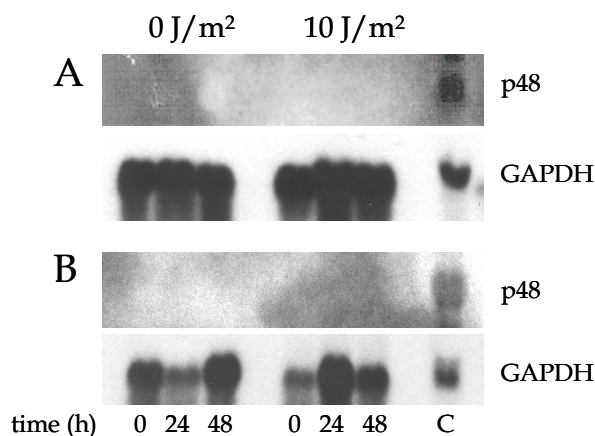


Figure 7. Northern blot analysis of p48 mRNA expression at various time points after UV-irradiation. RNA was isolated from primary cultures of cardiac myocytes (A) and fibroblasts (B) that either had been irradiated or mock treated. RNA (30 μ g per sample) was run on a 1% agarose/7.5% formaldehyde gel and transferred to a Hybond filter. p48 mRNA was detected by hybridization with a p48 cDNA probe. The presence of equivalent amounts of RNA in each lane was checked by rehybridization of the blots with a GAPDH cDNA probe. As a positive control RNA isolated from the testis of the mouse is used (C).

(Keeney et al., 1994; Nichols et al., 1996). To determine whether primary cultures of terminally differentiated cardiac myocytes and fibroblasts also lack the expression of p48, RT-PCR and northern blot analysis were performed. Northern blot analysis showed no p48 mRNA expression in myocytes and fibroblasts at various time points after UV-irradiation (Figure 7), a result which was confirmed by RT-PCR (data not shown).

Discussion

Already in the early 1920s anatomical studies emphasized the difficulty of detecting mitotic figures in myocytes (Karsner et al., 1925). Moreover experimental results of acute cardiac hypertrophy in rodents demonstrated the inability of these

cells to reenter the cell cycle (Chien and Olson, 2002). These observations were responsible for the creation of the dogma that, shortly after birth ventricular myocytes withdraw from the cell cycle permanently and are destined to die without further replication. Although most myocytes are terminally differentiated there is a small and continuously renewed sub population of cycling myocytes produced by the differentiation of cardiac stem-like cells of a yet unknown origin (Nadal-Ginard et al., 2003).

Extensive apoptosis will deteriorate heart function via uncompensated loss of myocytes, resulting in a loss of pump function of the whole heart and ultimately heart failure. To maintain proper function repair of DNA damage is crucial for these cells.

The capacity of terminally differentiated cardiac myocytes and proliferating cardiac fibroblasts of neonatal rats to carry out NER was examined employing UV light as a model genotoxic agent. Like established rodent cells both cardiac myocytes and cardiac fibroblasts are proficient in repair of UV-induced 6-4 PP in bulk DNA and deficient in repair of UV-induced CPD by GGR. The induction and repair of UV-induced CPD in transcriptionally active and inactive genes were analysed to determine the potency of cardiac myocytes and fibroblasts to carry out TCR of CPD. In both cell types the removal of CPD from the HPRT gene was confined to the transcribed strand, whereas both strands of the fragment encompassing the APRT sequence were repaired efficiently, a phenomenon also observed in the APRT gene in hamster cells (Vreeswijk et al., 1994). The lack of strand bias in the APRT region is best explained by the existence of another transcript running in the opposite direction of the APRT transcription unit as discussed previously (Vreeswijk et al., 1994). However, CPD repair in both strands of the c-mos gene was virtually absent in cardiac fibroblasts whereas cardiac myocytes exhibited significant removal of CPD from the transcribed strand of the c-mos gene. Since TCR is tightly coupled to transcription, the latter observation suggests that c-mos is an actively transcribed gene in primary cultures of cardiac myocytes but not in fibroblasts. Indeed, Leibovitch et al. (Leibovitch et al., 1990; Leibovitch et al., 1991) observed that c-mos transcripts and protein accumulate at increasing levels during post-natal development in cardiac tissue up till 12-13 days following birth, after which the levels start to decline to the low perinatal values. The primary cultures of cardiac myocytes and fibroblasts used in the present study were isolated from two-day-old rats and repair experiments were performed within the time period that c-mos expression was observed in post-natal cardiac tissue of the rat (Leibovitch et al., 1990; Leibovitch et al., 1991).

The efficient repair of CPD in transcriptionally active genes in myocytes and fibroblasts is in agreement with the low apoptotic response after UV-irradiation, as apoptosis inhibits both TCR and GGR in rodent cells (Van Sloun et al., 1999; Vreeswijk et al., 1998). Taken together, our results demonstrate that primary nondividing myocytes and proliferating fibroblasts exhibit TCR of UV-induced CPD, but poor GGR repair of this photolesion. Furthermore both cell types display efficient removal of 6-4 PP from the genome overall.

The repair pattern found in non-dividing myocytes and proliferating fibroblasts closely mimics the repair phenotype of other rodent cells such as primary mouse keratinocytes (van Oosten et al., 2000) and established hamster and mouse cell lines (Vreeswijk et al., 1998). The low efficiency of CPD removal by GGR has been attributed to the absence of expression of the p48 gene (Keeney et al., 1994; Tang et al., 2000). This gene has been shown to be mutated in XP patients belonging to complementation group E (Nichols et al., 1996). There is convincing evidence that the p48 gene product forms a complex with p125 (termed DDB) and it has been proposed that this complex facilitates the recognition of CPD photolesions and other DNA lesions in their chromatin context by the NER proteins involved in GGR, whereas TCR is unaffected by the level of p48 expression. The absence of p48 expression in UV-irradiated myocytes and fibroblasts is consistent with the poor repair of CPD by GGR.

The data from our study together with the data presented by Guo et al (Guo et al., 1998), indicating that primary cultures of non-dividing rat hepatocytes (parenchymal cells) displayed TCR of UV-induced damage, demonstrate that TCR of DNA damage by the NER pathway occurs in primary cells that are terminally differentiated. This observation is consistent with the observation of Islas and Hanawalt (Islas and Hanawalt, 1995) who demonstrated that TCR of UV-induced CPD is still present in cell lines after in vitro induction of differentiation in postmitotic, terminally differentiated cells. Thus differentiation is not associated with a loss in TCR of UV-induced CPD as was suggested by the initial studies of Bill et al (Bill et al., 1991) and Ho and Hanawalt (Ho and Hanawalt, 1991). Interestingly examining repair in the NT2-hNT model system for differentiating human neurons Nospikel and Hanawalt observed a phenomenon which they termed differentiation associated repair (DAR) (Nospikel and Hanawalt, 2000). They demonstrated a marked decrease in GGR of CPD in non-transcribed genes upon differentiation of hNT neurons. Repair of transcribed genes, on the other hand, remained fully functional in the differentiated hNT neurons. Besides, unlike observations made in rodent cell lines and the mouse epidermis, repair in the non-

transcribed strand was also proficient. More recently they confirmed the existence of DAR in human primary neurons (Nospikel and Hanawalt, 2002). The data from the present study together with the data of Guo (Guo et al., 1998) and Islas (Islas and Hanawalt, 1995) do not point to the existence of DAR in terminally differentiated cells. From this we can deduce that DAR is not a hallmark for terminal differentiation. Nevertheless we have to take into account that DAR is observed in terminally differentiated human cells and not in terminally differentiated rodent cells and therefore, DAR might be species specific rather than cell type specific. Our results and data from the literature suggest that the expression of p48 (Tan and Chu, 2002) and consequently the efficiency of repair of certain types of DNA lesions by GGR differs among the various tissues of the rat and is not dependent on terminal differentiation status. The differences in GGR among the various rat tissues might have important consequences for tissue specific cytotoxic and mutagenic effects of DNA damaging agents, i.e. tissues not expressing GGR might be more prone to DNA damage mediated mutations when stimulated to proliferate.

Acknowledgements

The authors wish to thank Mr. P. de Jong of the Animal Care Facilities and Mrs. E.J.M. van der Valk for technical assistance. This study was supported by the Dutch Heart Foundation # 97.176.

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Chapter four

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5

**Separation of neonatal rat ventricular
myocytes and non-myocytes by centrifugal
elutriation.**

Chapter five

Separation of neonatal rat ventricular myocytes and non-myocytes by centrifugal elutriation.

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Pflügers Archive - European Journal of Physiology 444: 452-456, 2002

Abstract

The preparation of pure cardiac myocyte cultures from neonatal rats is hampered by the presence of non-myocytes, that are capable to proliferate during culturing, thereby causing a progressive decrease in the proportion of myocytes. In order to obtain myocyte cell suspensions of high purity, a method based on centrifugal elutriation was developed.

Cardiac cells, isolated from neonatal rat heart ventricles, were subjected to elutriation using flow rates that increased step-wise from 20 to 80 ml/min. The cell fraction obtained at 80 ml/min consisted for 68-90% of myocytes. Still, upon culturing, the remaining non-myocytes proliferate, causing the proportion of myocytes to decrease to $60 \pm 2\%$ at day 5.

A second elutriation protocol was developed in which myocytes and non-myocytes were separated after a period of co-culturing for 4-5 days. By this approach a fibroblast-rich cell fraction ($87 \pm 5\%$) and a myocyte-rich cell fraction ($82 \pm 6\%$) were obtained.

In conclusion, centrifugal elutriation creates the opportunity to separate neonatal rat myocytes from non-myocytes, either freshly isolated or after a period of culturing. Particularly, cell separation after a period of culturing ventricular cells offers an advantage to analyse experimental effects on myocytes and non-myocytes separately.

Key words

myocytes, cell separation, neonatal rats, primary cell cultures, centrifugal elutriation

Introduction

Primary cultures of neonatal rat heart myocytes are often applied as a model to study heart cell physiology and biochemistry (Gardner and Schultz, 1990; Li et al., 1999; Sadoshima and Izumo, 1993). Isolation and culturing of these cells result in cultures of non-dividing, spontaneously beating myocytes. A major practical

problem is the presence of non-myocytes, mostly fibroblasts, that proliferate and therefore cause a decrease in the final proportion of myocytes (Lokuta et al., 1994; Van der Laarse et al., 1989). In order to obtain myocyte cultures of high purity, several methods have been applied, including differential pre-plating based on the preferential attachment of fibroblasts to culture flasks (Blondel et al., 1971), density gradient centrifugation (Flanders et al., 1995; Harada et al., 1998), application of culture medium deprived of serum (Shields et al., 1988), and a combination of techniques (Roberts et al., 1992). In several studies, bromodeoxyuridine or arabinosyl-cytosine have been added to the culture medium, to prevent proliferation of residual non-myocytes (Boerma et al., 2002; Sei et al., 1992; Simpson and Savion, 1982).

In a previous study, centrifugal elutriation was described to obtain relatively pure cultures of neonatal rat ventricular myocytes (Diamant et al., 1989). This method is based on separation of cells by differences in cell size and density (Sanders and Soll, 1989). In the present study this elutriation method was further developed, resulting in two protocols: protocol 1 was used with the purpose to start culturing with a high proportion of myocytes and protocol 2 was used to separate myocytes and non-myocytes after an experimental treatment of several days, to allow analysis of both cell populations under identical experimental conditions.

Materials and Methods

Preparation of neonatal rat cardiac myocytes and fibroblasts

Ventricular myocytes and fibroblasts were isolated from two-day-old neonatal Wistar rats as described earlier (Van der Laarse et al., 1979). Hearts were excised from rats anaesthetised with ethyl ether and transferred to solution A (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 5.6 mM D-glucose, 20 mM HEPES and 0.02% Phenol red, pH 7.4), after which ventricles were separated and minced into small fragments. Tissue fragments were further dissociated by incubating them twice with an enzyme solution containing 450 U collagenase type I (Worthington Biochemical Corporation, Freehold, NJ) and 14 U DNase (Worthington Biochemical Corporation) per ml solution A in a shaking water bath at 37°C for 30 min. Pooled cell suspensions were cooled on ice and pelleted by centrifugation (15 min, 300g). If purified by elutriation, cells were resuspended in Hank's salt solution (Life Technologies, Rockville, MD) supplemented with BSA

(0.6%) and HEPES (6.7 mM) (Hank's+). If purified by pre-plating, cells were resuspended in growth medium consisting of Ham's F10 supplemented with 10% fetal bovine serum (FBS, Life Technologies), 10% horse serum (HS, Life Technologies) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The experiments had the approval of the Animal Experiments Committee of the institution, installed by Leiden University, according to Dutch law.

Separation of myocytes and non-myocytes by elutriation, prior to culturing (protocol 1)

Removal of erythrocytes by Nycodenz gradient centrifugation

Five ml of cell suspension in Hank's+ were mixed with 7.3 ml Nycodenz buffer (containing: 9.2 mM HEPES, 6.7 mM KCl, 1.2 mM CaCl₂ and 5.5 mM NaOH, pH 7.6) and 2.1 g Nycodenz (Axis-Shield, Oslo, Norway) and centrifuged at 1500g for 15 min at RT. The layer containing the cardiac cells was collected and resuspended in 5 ml Hank's+ at 4°C.

Separation of cardiac myocytes and non-myocytes by elutriation

The method to separate myocytes and non-myocytes by elutriation was adapted from Diamant et al. (Diamant et al., 1989). The suspension of cardiac cells, obtained by Nycodenz gradient centrifugation, was injected into a rotating Beckman JE-6B elutriator rotor adapted to a Beckman J2-21 high speed centrifuge, that was kept at a temperature of 4°C. The elutriation system was equipped with a Sanderson chamber and the rotor speed was adjusted to 3200rpm (1216g). A pump with a speed controller was used to establish the counterflow force with ice-cold Hank's+. Fractions of 150 ml were collected at each flow rate.

Cell culturing

The collected cells were spun down for 10 min at 300g and washed three times with 1 ml sterile PBS at 4°C. Cells were resuspended in DMEM and Ham's F10 (1:1) supplemented with 5% HS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in Primaria® culture flasks (Becton Dickinson, Franklin Lakes, NJ). Cell cultures were incubated at 37°C at 5% CO₂ in a humidified atmosphere. Culture medium was changed every day.

Separation of cardiac myocytes and non-myocytes after cell culturing (protocol 2)

Pre-plating

Myocytes were separated from non-myocytes by using a selective adhesion technique: the rate of attachment of non-myocytes to the culture dishes is higher than that of the myocytes [1]. After 60 min, non-adherent cells (mainly cardiac myocytes) were collected and transferred to Primaria® culture disks (Becton Dickinson). The myocyte-enriched cell population was cultured in DMEM and Ham's F10 (1:1) supplemented with 5% HS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cell cultures were incubated at 37°C at 5% CO₂ in a humidified atmosphere. Culture medium was changed after 4h and after each 24h.

Separation of cardiac myocytes and non-myocytes by elutriation

Following a culturing period of 4-5 days cells were trypsinized for 15 min at 37°C and collected in 5 ml Hank's+ at 4°C. This concentrated cell suspension was injected into a rotating Beckman JE-6B elutriator as described above.

Cell viability

After elutriation, cell viability was determined by trypan blue exclusion.

Characterizing the cells by FACS analysis

Cells fixed in 1% formaldehyde were permeabilized using digitonin (20 µg/ml in PBS). Aspecific binding was prevented by incubation in PBS containing 1% FBS and 1% goat serum. Equal fractions were incubated with monoclonal mouse anti-myosin antibody (Sigma-Aldrich, St. Louis, MO, clone NOQ 7.5.4D) or monoclonal mouse anti- α -actinin antibody (Sigma-Aldrich, clone EA-53), which are both specific for myocytes, for 30 min on ice. Thereafter, cells were incubated with FITC-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) for 30 min on ice. Cells were resuspended in PBS containing 1% FBS and analyzed on a FACScan flow cytometer (Becton Dickinson). Data analysis was performed by using PC-LYSIS software (Becton Dickinson).

Characterization of cardiac myocytes by immunostaining

All steps were performed on ice. Following removal of culture medium monolayers of cells were fixed in 1% formaline in PBS for 30 min. Subsequently, cells were permeabilized in 0.1% Triton X-100 for 30 min and incubated with monoclonal mouse anti- α -actinin antibody (Sigma, clone EA-53) for 3h at 4°C. Thereafter, cells were incubated with FITC-conjugated rabbit anti-mouse IgG antibody (Sigma) for 30 min. To visualize the nuclei, cells were counterstained with Hoechst 33342 for 10 min.

Results

Centrifugal elutriation of freshly isolated primary cardiac cells (protocol 1)

After isolation of cells from neonatal rat heart ventricles by collagenase treatment and Nycodenz gradient centrifugation, the cell suspension was elutriated, applying flow rates that increased step-wise from 10 to 80 ml/min. The percentages of myocytes (i.e. myosin and α -actinin expressing cells), as determined by FACS analysis, are shown in figure 1A.

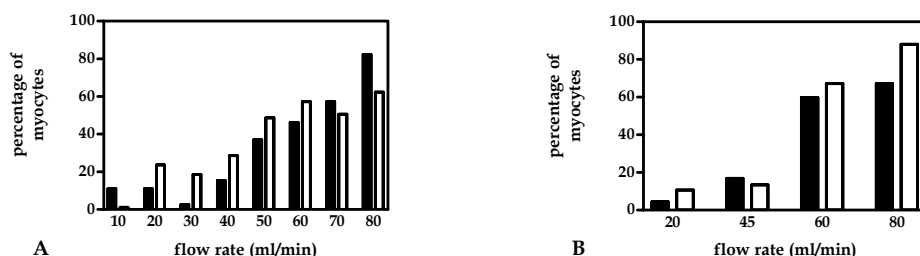


Figure 1. Percentages of myocytes in A) consecutive cell fractions using flow rates between 10 and 80 ml/min and B) four cell fractions using flow rates of 20, 45, 60 or 80 ml/min, obtained by elutriation protocol 1. Open and solid bars represent two parallel experiments.

The fractions obtained using flow rates of 10 and 20 ml/min contained mainly cellular debris. The fractions obtained using flow rates of 30 and 40 ml/min primarily consisted of non-myocytes. In the fractions collected using flow rates of 50, 60 and 70 ml/min a mixture of myocytes and non-myocytes was present and the highest flow rate (80 ml/min) yielded a cell suspension with a high percentage of myocytes.

Figure 2 shows typical cell cultures of the fractions, obtained by a flow rate of 40, 60 and 80 ml/min, stained for α -actinin. The α -actinin positive cells are recognized as myocytes by the cross-striated myofilaments.

Based on these results, flow rates of 20 ml/min (to discard cellular debris), 45 ml/min (to obtain a non-myocyte fraction), 60 ml/min (to further discard non-myocytes) and 80 ml/min (to obtain myocytes) were chosen. The percentages of myocytes in these fractions are shown in figure 1B. By applying the highest flow rate (80 ml/min), a myocyte proportion between 68% and 90% was obtained.

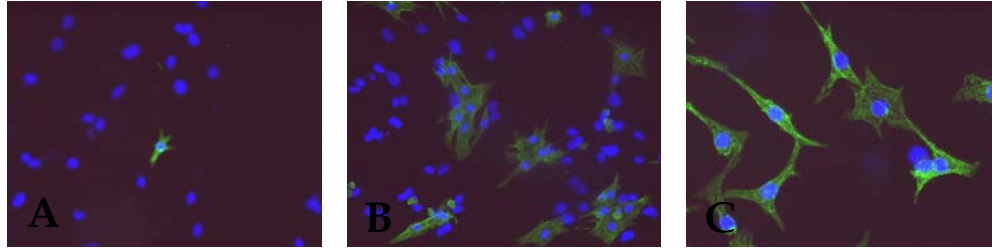


Figure 2. Ventricular cells, stained for α -actinin (myocytes) and counterstained with Hoechst 33342 (nuclei). Cells were obtained by flow rates of A) 40 ml/min, B) 60 ml/min and C) 80 ml/min (protocol 1) and cultured for 1 day.

Culturing of cells after centrifugal elutriation

During 5 days of culturing of the different cell fractions, obtained by elutriation protocol 1, percentages of myocytes were determined (figure 3). Total numbers of myocytes in these cultures did not change (data not shown). Apparently, a small number of non-myocytes proliferated, thereby causing a progressive reduction in the percentage of myocytes in each cultured cell fraction.

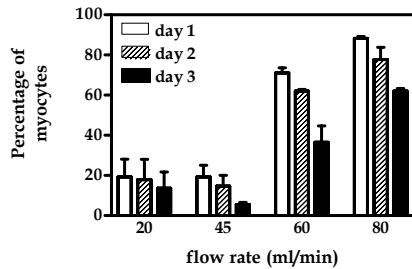


Figure 3. Percentages of myocytes obtained by protocol 1, immediately after elutriation (day 1) and at 3 and 5 days after culturing. Means $\pm$ SD, N=3.

Centrifugal elutriation of cultured cells (protocol 2)

Ventricular cells were isolated by collagenase treatment and myocyte-enriched cell cultures were obtained by differential pre-plating. After 4-5 days of culturing, cells were trypsinized and centrifugal elutriation was performed, applying flow rates that increased step-wise from 20 to 80 ml/min. The percentages of myocytes in the subsequent fractions are shown in figure 4A. The fractions obtained using flow rates of 20 and 30 ml/min mainly contained cellular debris, of which a part was positive for myosin or α -actinin. The fractions obtained using flow rates of 40, 50 and 60 ml/min, primarily consisted of non-myocytes. The fractions collected using flow rates of 70 and 80 ml/min contained a mixture of myocytes and non-myocytes. The cell fraction that remained in the elutriation chamber contained the highest percentage of myocytes.

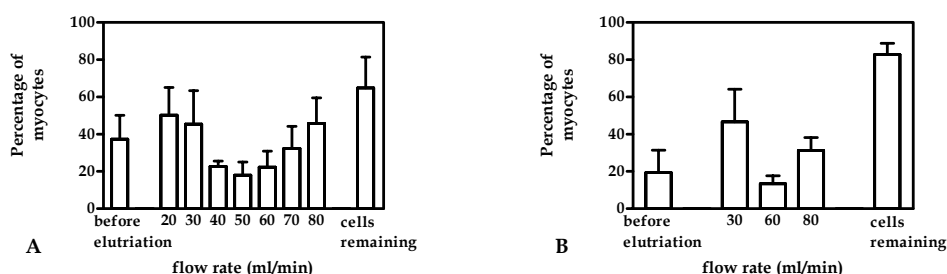


Figure 4. Percentages of myocytes obtained by elutriation protocol 2 in A) consecutive cell fractions using flow rates between 10 and 80 ml/min (means $\pm$ SD, N=3) and B) cell fractions using flow rates of 30, 60 and 80 ml/min and cells remaining in the elutriation chamber (means $\pm$ SD, N=4).

No hearts		Injected ($\times 10^6$)	60 ml/min ($\times 10^6$)	Remaining in chamber ($\times 10^6$)
15	Myocytes	10.0 (100%)	-	2.2 (22%)
	Non-myocytes	14.0 (100%)	7.6 (54%)	-
15	Myocytes	11.0 (100%)	-	2.3 (21%)
	Non-myocytes	14.0 (100%)	6.3 (45%)	-
10	Myocytes	8.0 (100%)	-	2.0 (25%)
	Non-myocytes	23.0 (100%)	14 (61%)	-
10	Myocytes	2.0 (100%)	-	0.6 (30%)
	Non-myocytes	4.8 (100%)	1.9 (40%)	-

Table 1. Relative yield of cardiac cells in the myocyte and non-myocyte rich fractions after elutriation by protocol 2, in 4 parallel experiments.

Based on these results, flow rates of 30 ml/min (to discard cellular debris), 60 ml/min (to obtain a non-myocyte fraction), and 80 ml/min (to further discard non-myocytes) were chosen. The cell fraction that remained in the elutriation chamber contained a high percentage of myocytes ($82 \pm 6\%$, figure 4B), with a yield of 20-30% of the total number of myocytes injected into the elutriation chamber (table 1).

Discussion

Primary cultures of neonatal cardiac myocytes are often utilized to examine a wide variety of physiological and biochemical properties of cardiac cells. These cell cultures include a population of dividing non-myocytes, mainly represented by fibroblasts. Primary cultures of myocytes are typically used for experiments within 4-5 days. Without any intervention, remaining fibroblasts proliferate and reduce the proportion of myocytes. Several methods have been applied to obtain myocyte cultures of high purity. Many of these methods have drawbacks, either due to the application of toxic compounds or due to the relatively low final percentage of myocytes after 4-7 days of culturing (Lokuta et al., 1994). In the present study it is shown that by the use of centrifugal elutriation a cell population of myocytes of high purity can be obtained. In 1989 Diamant et al. (Diamant et al., 1989) first applied this method to purify cultures of myocytes from neonatal rat hearts, obtained by enzymatic digestion. A myocyte fraction of high purity was obtained, although at a lower flow rate than used in the present study. This difference could be due to a lower rotor speed of the centrifuge (2000 rpm, compared with 3200 rpm in the present study) and the application of a different separation chamber, the shape of which affects the properties of the separation procedure (Lutz et al., 1992). Apparently, optimal conditions for separation highly depend on the experimental procedures and equipment. Diamant et al. (Diamant et al., 1989) showed that upon elutriation, the proportion of myocytes in the cell cultures remains high even after 48 hours of culturing. From the present study it is concluded that after a longer period of culturing (4-5 days) the small number of non-myocytes is responsible for a reduction of the final proportion of myocytes.

To be able to separately analyze myocytes and non-myocytes after an experiment, a second elutriation protocol (protocol 2) was developed, in which myocytes and non-myocytes were separated after a period of co-culturing for 4-5 days. The advantage of this method is that myocytes can be cultured in the presence of non-

myocytes, allowing interaction between both cell types, which reflects the *in vivo* situation in the rat heart. By applying this elutriation protocol relatively pure cell populations of myocytes and non-myocytes were obtained.

When the counterflow forces used in the two elutriation protocols are compared, several differences become apparent. In elutriation protocol 1 myocytes were removed from the elutriation chamber at a counterflow rate of 80 ml/min. However, when applying protocol 2, most myocytes remained in the elutriation chamber when using the same counterflow rate (80 ml/min). Analogously, to obtain a non-myocyte fraction, in protocol 2 a higher counterflow rate than in protocol 1 was necessary (60 ml/min and 45 ml/min, respectively). Apparently, the size of the cells has increased during culturing. This is confirmed by studies, in which myocyte cell surface area, cellular volume and protein content increased during culturing in medium supplemented with serum (1-10%) (Marino et al., 1987; Simpson et al., 1982; Starksen et al., 1986; Simpson et al., 1982; Starksen et al., 1986).

A flow rate higher than 80 ml/min has not been used, as higher counterflow forces may induce hydrodynamic disturbances that will damage the cells. Taken together, these results demonstrate that centrifugal elutriation is a powerful method to obtain separate populations of myocytes and non-myocytes with high purity. Application of this method immediately after isolation creates the opportunity to study the effect of *in vivo* treatments on myocytes and non-myocytes separately. Furthermore, cell separation after a period of culturing myocytes in the presence of non-myocytes offers a major advantage to analyse experimental effects on myocytes and non-myocytes separately. Since all steps are performed at 4°C and no toxic compounds are applied, this technique offers many opportunities for cellular and molecular research, among others analysis of cell specific gene expression.

Acknowledgements

The authors thank Mr. Kar Kruit of the Division of Biopharmaceutics, Leiden University, for technical support.

C.G.C. van der Wees is a recipient of a grant (#97.176) from the Netherlands Heart Foundation. M. Boerma is a recipient of a grant (#9.0.13) from the Interuniversity Research Institute of Radiopathology and Radiation Protection.

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6

Is mechanical stress the stimulus for hypertrophy? A micro array analysis of cardiac myocytes and fibroblasts

Chapter six

Is mechanical stress the stimulus for hypertrophy? A micro array analysis of cardiac myocytes and fibroblasts

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Submitted

Abstract

Several stressful conditions cause growth of the heart that exceeds growth that normally occurs during development of the organism, a phenomenon that is termed cardiac hypertrophy. Although compensatory growth of myocardial tissue is beneficial initially, since it reduces wall stress and permits normal cardiovascular function, transition to heart failure may occur. In cardiac adaptation to hemodynamic overload (force-length mechanism and homeometric auto regulation); mechanical forces activate cellular signaling mechanisms that initiate hypertrophic growth of cardiac myocytes. We set out to characterize the myocardial hypertrophic response to myocardial stretch. The present study describes a genome wide screening of changes in gene transcription due to mechanical stress (MS) of cardiac myocytes and fibroblasts. In addition to investigation of changes in transcription of genes that encode proteins involved in apoptosis, DNA damage was measured using alkaline unwinding followed by the determination of the amount of single stranded DNA (ssDNA) breaks in cell cultures exposed to mechanical stress for 24 h. However, exposure of cardiac cells to MS for 24 h did not induce an increase in ssDNA fragments.

Affymetrix micro arrays, analyzed with Linear modeling on Probe Level, were used to determine gene expression patterns at 24 h after the onset of treatment. MS was associated with up-regulation of 145 genes and down-regulation of 15 genes. Genes encoding proteins involved in production and degradation of the extracellular matrix (ECM), growth factors and growth factor receptors, as well as proteins involved in energy metabolism, contractile filaments and proteins involved in calcium homeostasis have been observed to be predominantly up-regulated by 24 h of MS. An interesting finding is the MS-induced up-regulation of genes encoding proteins involved in fatty acid synthesis that is accompanied by up-regulation of proteins of the glycolysis pathway. Therefore we argue that stimulated synthesis of fatty acids is intended for synthesis of phospholipids and sterols, both being the building blocks of plasma membranes, which synthesis should meet the demand of growing myocytes.

The MS-induced changes in expression of genes described in this study indicate that mechanical stress of cardiac cells can be considered as the initiation factor of the hypertrophic response in those cells.

Introduction

Growth of the heart is a dynamic process that occurs during embryogenesis, postnatal development, maturity and senescence. Myocardial growth may also be a response to environmental and pathologic conditions. At the cellular level cardiac growth occurs as a consequence of the interplay between hyperplasia (increase in cell number) and hypertrophy (increase in cell size). The relative importance of each of these processes depends on cell type, developmental stage and the nature of the growth stimulus. Although cardiac myocytes comprise only 25% of the total cell number of the heart, they make up > 70% of the adult cardiac mass (Vliegen et al., 1991; Zak, 1973). The prevailing view is, although still actively debated, that adult mammalian ventricular myocytes are terminally differentiated cells. However, they remain capable to undergo karyokinesis, without entering cytokinesis (Tamamori-Adachi et al., 2003). Consequently, the increase in cardiac mass during normal post natal cardiac growth is mainly the result of an increase in size of terminally differentiated cardiac myocytes.

Several stressful conditions cause growth of the heart that exceeds general cardiac growth that occurs during normal development of the organism, a phenomenon that is termed cardiac hypertrophy. Compensatory growth of myocardial tissue is initially beneficial since it reduces wall stress and permits normal cardiovascular function. However, if the stimulus for pathologic hypertrophy is too intense or prolonged, the adaptation and functional alterations become insufficient to normalize wall stress, leading to a transition from compensated hypertrophy to heart failure (Bing, 1994; Colucci, 1997).

Mechanical forces play a major role in cardiac adaptation to hemodynamic overload (Cooper, 1997). In addition to triggering the force-length mechanism and homeometric autoregulation, mechanical forces activate cellular signaling mechanisms involved in hypertrophic growth of cardiac myocytes (Ruwhof and Van der Laarse, 2000; Cooper, 1990; Sadoshima et al., 1992; Sadoshima and Izumo, 1997). The view that mechanical stress itself is the primary factor for cardiac hypertrophy in response to hemodynamic overload is supported by several *ex vivo* experiments. For example, increased cardiac load stimulated protein synthesis in isolated hearts (Kira et al., 1984) and stretching cultured cardiac myocytes stimulated protein synthesis and induced alterations in gene expression (Kira et al., 1994; Mann et al., 1989; Ruwhof et al., 2001; Sadoshima et al., 1992; Vandenberg et al., 1995), effects that are similar to those observed in the *in vivo* heart in response

to hemodynamic overload. Yet little is known about how individual cardiac cells transduce mechanical signals into a myocardial hypertrophic response.

The present study is performed to examine the consequences of mechanical stress on cardiac cells in vitro, in terms of changes in gene expression. Previous studies on gene expression after exposure of in vitro cultured cardiac cells to mechanical stress, focused on early changes of selected genes, analysed by Northern blotting and RT-PCR. In this study we performed genomic wide analysis of changes in gene transcription due to mechanical stress of cardiac myocytes and fibroblasts.

These genome wide expression profiles were correlated with the induction of apoptosis, a process known to be induced by mechanical stress (Cheng et al., 1995). DNA damage was measured by quantification of single stranded DNA (ssDNA) in cell cultures exposed to mechanical stress for 24 h.

Materials and methods

Cell culturing

Ventricular myocytes and fibroblasts were isolated from two-day-old neonatal Wistar rats as described earlier (van der Wees et al., 2003). By applying a pre-plating method, cultures enriched for cardiac myocytes and cultures of fibroblasts were obtained. After 2 days of culturing with 5% horse serum (HS), culture medium of the cardiac-myocyte enriched cultures were replaced by media containing 2.5% HS. Twenty-four h later these cultures were subjected to mechanical stress. Three days after isolation, cultures of fibroblasts were subcultured for 6 days in medium containing 10% fetal bovine serum (FBS). Then, the culture medium was replaced by medium containing 2.5% FBS. One day later, confluent cell cultures were subjected to mechanical stress.

The experiments had the approval of the Animal Experiments Committee of the Leiden University Medical Center.

Mechanical stress experiments

The Flexercell® strain unit (Flexcell Int. Corp., Dunn Laboratories, Asbach, Germany) is a modification of the unit initially described by Banes et al. (Banes et al., 1985) and consists of a computer controlled vacuum unit and a base plate to hold eight six-well culture plates. Cells were grown on Flex I® culture plates that

have flexible bottoms of a silicone elastomer coated with collagen I (Flexcell Int. Corp., Dunn Laboratories, Asbach, Germany). When vacuum is applied below the membranes, the membranes are pulled down and stretched. Since the cells are firmly attached to the membranes the deformation of the membranes stretches the cell. Cells were submitted to cyclic stretch (1Hz; per cycle 0.5 s elongation and 0.5 s relaxation) with a maximal elongation of 20% in a humidified incubator with 5% CO₂ at 37°C. As a control, cells derived from the same pool of cells were grown on the same Flex I ® culture plates and cultured under identical conditions, except that they were not submitted to stretch.

Cell harvesting and determination of percentage of myocytes

After receiving mechanical stress for 24 h, cells were collected by trypsinisation and the percentages of myocytes and fibroblasts present in the cell culture were determined by flow cytometric analysis of myosin expression as described before (Boerma et al., 2002). Briefly, cells fixed in 1% formaldehyde were permeabilized using digitonin (20 µg/ml in PBS). Aspecific binding was prevented by incubation in PBS containing 1% FBS and 1% goat serum. Fractions were incubated with monoclonal mouse anti-myosin antibody (Sigma-Aldrich, St. Louis, MO, clone NOQ 7.5.4D), which is specific for myocytes, for 30 min on ice. Thereafter, cells were incubated with FITC-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) for 30 min on ice. Cells were resuspended in PBS containing 1% FBS and analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data analysis was performed by using PC-LYSIS software (Becton Dickinson).

RNA Extraction and Affymetrix GeneChip Hybridization

From three independent mechanical stress experiments RNA was isolated and labeled. Total RNA was isolated by using a RNeasy® kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. 10-14 µg of total RNA was used for labelling, with the quantities of total RNA from stressed and control cells being equal per experiment. Double stranded cDNA was synthesised using the Gibco BRL Superscript system (Invitrogen, Carlsbad CA, USA). Briefly, single stranded cDNA was synthesised using Superscript II reverse transcriptase and T7-oligo(dT)₂₄ primers at 42°C for 1 h. Next, double stranded cDNA was obtained by

using *E. coli* DNA polymerase I, DNA ligase and RNase H (GIBCO, Life technologies, Rockville, USA) at 16°C for 2 h, followed by T4 DNA polymerase at 16°C for 5 min. Following clean up with a phase lock gel (Qiagen) the resultant ds-cDNA was used for in vitro transcription (IVT). Ds-DNA was transcribed using the MEGAScript T7® kit (Ambion, Austin, TX, USA) in the presence of biotin-labeled UTP and CTP (PerkinElmer, Foster City, CA, USA). Following clean up with the RNeasy kit (Qiagen), the biotin-labeled IVT-RNA was fragmented in a buffer containing 40 mmol/L Tris-acetate, pH 8.1, 100 mmol/L sodium-acetate and 30 mmol/L magnesium-acetate, at 94°C for 35 min.

Hybridization of IVT-RNA

For the analysis of roughly 9000 rat genes the GeneChip RG-U34A array (Affymetrix, Santa Clara, CA, USA) was used. The biotin-labeled IVT-RNA was hybridized to the arrays according to the manufacturer's instructions, at 45°C for 16 h. Following hybridization the chips were washed in a GeneChip Fluidics Station 400 with a non-stringent wash buffer at 25°C followed by a stringent wash buffer at 50°C. Subsequently the arrays were stained with a streptavidin-phycoerythrin complex, and the intensities were determined with a GeneChip scanner, controlled by GeneChip software. The intensities were background corrected using GCRMA -Robust Multi-Array expression measure using sequence information- (Wu and Irizarry, 2004) and normalized at the probe level by VSN - Variance stabilization and calibration for micro array data (Huber et al., 2002).

Data analysis

The GeneChip array, RG-U34A, designed for the rat, consist of 16 probe-pairs per transcript, with no or minimal overlap. In the present analysis only the perfect match signal was used since the effect of the mismatch values on the final outcome are shown to be marginal (Chu et al., 2002). For each condition three independent experiments were performed. To describe the signal of every perfect match (PM) probe, the following linear model was used: $\text{signal} \sim P + T + E + C + PE$, based on a method described before (Chu et al., 2002). In our model the symbols P, T, E, C and PE represent the effects of probe, treatment (stretched or non-stretched), experiment (1, 2 or 3), cell-population (fibroblast or myocyte-enriched) and the interaction between probe and experiment, respectively. Subsequently analysis of

variance was applied on the treatment effect to determine the p-value for each probe set. The p-values were corrected for multiple testing using the Benjamini-Hochberg step-up procedure (Benjamini and Hochberg, 1995) which yielded q-values for the false discovery rates (FDR). The FDR level of control was set to 0.005, equivalent to selecting genes with a q-value < 0.005 (keeping up- and down-regulated genes separate by the sign of the treatment coefficient). Some genes are represented by more than one probe set. A gene was considered to be differentially expressed when at least 50% of the probe-sets, representing that gene, showed significant up- or down-regulation.

To assess the goodness-of-fit of the linear model that was used to describe PM signals R^2 was calculated for every probe set, using the following standard formula: $R^2 = 1 - \sum R_i^2 / \sum (\text{signal}_i - \text{mean signal})^2$, where R_i = observed-fitted value for signal_i and mean signal = the mean of observed signal_i .

To compare data on differentially expressed genes with micro array data in literature on species other than rat, RESOURCER of the Institute of Genomic Research (available at: <http://www.tigr.org/tigr-scripts/magic/r1.pl>) was used.

RNA isolation and semi-quantitative PCR

Total RNA isolation, cDNA synthesis and semi-quantitative PCR were performed as described before [40]. In short, total RNA was isolated using an RNeasy® kit (Qiagen). Following DNase (Amersham Pharmacia Biotech, Uppsala, Sweden) treatment, cDNA was synthesised using M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA) and oligo(dT) primers (Amersham). Semi-quantitative PCR was performed, using the following primers and annealing temperatures: Tenascin C sense: CGA CAG TTT TGT TAT CAG GAT CAG, Tenascin C antisense: GGC ACA TAA GTA ATC CGG AAA T, 60 °C; Biglycan sense: CAA CAA CCC TGT GCC CTA CT, Biglycan antisense: GGT GT GCT TCT TTG CTT CC, 65 °C. A competitive PCR was performed on GAPDH to correct for differences in cDNA concentrations. After agarose gel electrophoresis, intensities of PCR product bands were determined by Scion image analysis software (Scion Corporation, Frederick, MD, USA).

Preparation of samples for the sandwich ELISA

Mechanical stress has been reported to induce apoptosis and consequent DNA damage. A sandwich ELISA, as described by Timmerman et al. (Timmerman et al., 1995), was performed to examine whether MS results in single stranded DNA (ssDNA) breaks. For this purpose, cells grown on stretch plates were harvested by trypsinisation at various time points after the onset of cyclic mechanical stretch and resuspended in ice-cold PBS at a density of 0.5×10^6 cells/ml. Subsequently DNA was partially unwinded by gently incubating 30 μ l of cells with 200 μ l alkaline buffer pH 12.3 solution (1.3 M NaCl adjusted to pH 12.3 with 1 M NaOH) in a polystyrene clustertube (Costar Europe Ltd., Badhoevedorp, The Netherlands). This mixture was kept at 20°C for exactly 6 min after which it was neutralized by adding 35 μ l 250 mM NaH₂PO₄. DNA was fragmented by sonication and stored at -20°C until the sandwich ELISA was performed. Staurosporine, a known protein kinase inhibitor and inducer of apoptosis, was used in this study as a positive control. Addition of 500 nM staurosporine resulted in a massive apoptotic response after 4-6 hours (Persoon-Rotherth et al., 2002).

Sandwich ELISA

A polystyrene microtiter plate was coated with D1B, an antibody directed against ssDNA. The 12 wells in the first row of this microtiter plate were filled with 120 μ l PBS and the other rows with 70 μ l PBS/well. Of each DNA sample (a mixture of single- and double-stranded fragments) 20 μ l aliquots were added to two neighboring cells of the first row. Two other 20 μ l aliquots were pipetted into two neighboring wells after pretreatment of 25 μ l of the sample in cluster tubes with 200 μ l pH 12.3 solution (1.3 M NaCl adjusted to pH 12.3 with 1 M NaOH) for two min, followed by addition of 25 μ l 250 mM NaH₂PO₄, to obtain a 100% ssDNA sample. Immediately after addition of the samples to the first row, twofold serial dilutions of these solutions were made in the following six rows, the last row was used to determine the background (adsorption of DNA to the wall during the period of dilution is marginal). The plate was left at room temperature for at least 5 min under continuous vibration. The wells were washed three times with PBS containing 0.05% Tween 20 and incubated with 100 μ l of D1B alkaline phosphatase conjugate, diluted 1:15000 in PBS containing 0.05% SDS and 5% FCS, for 5 min at room temperature under continuous vibration. Following repeated washes with PBS/ 0.05% Tween 20 and once with 0.1 M diethanolamine (pH 9.8), 0.2 mM 4-

methyumbelliferyl phosphate was added. After incubation for 1 h at 37°C fluorescence was recorded with a Fluoroskan (Eflab, Finland). Both microtiter plates precoated with D1B and the D1B alkaline phosphatase conjugates were kindly provided by Dr. G.P. van der Schans (T.N.O. Nutrition and Food Research Institute, Department of Genetic Toxicology, Rijswijk, The Netherlands).

Calculation of the percentage of DNA single-strandedness

To calculate the fraction of single-strandedness in a particular sample, the ratio of the fluorescence of the DNA dilution to that of the corresponding completely 100% ssDNA dilutions, both corrected for background fluorescence (fluorescence in wells without DNA), is divided by a factor of 10 (because of the pre-dilution of the 100% ssDNA sample), as follows:

$$\% \text{ single strandedness} = \frac{(F \text{ sample} - F \text{ background})}{10 \times (F \text{ 100\%ss} - F \text{ background})} \times 100\%$$

Results

Percentages of cardiac myocytes in the cultures

After receiving mechanical stress (MS) for 24 h the percentages of myocytes and fibroblasts present in the cell cultures were determined by flow cytometric analysis. Although there is a slight difference in percentage of cardiac myocytes between the experiments, the percentages of myocytes and fibroblasts in control cultures and in cultures that underwent MS were equal per experiment (table 1), demonstrating that the small difference in percentage of cardiac myocytes between the experiments was the result of variations due to cell isolation. The fibroblast cell populations demonstrated a high purity ($95 \pm 2\%$) in both the control and the MS cell populations.

Table 1. Percentages of cardiac myocytes in the myocyte-fibroblast cell populations in three parallel experiments.

experiment #	% myocytes control cells	% myocytes MS cells
1	60.4	59.0
2	53.6	54.8
3	48.6	47.8

Accuracy of the linear model

To demonstrate the accuracy of the linear model that was used to describe the PM probe signals, figure 1 shows the distribution of correlation coefficients (R^2) that were determined between the signals calculated by the model and the actual PM signals obtained. The majority of probe-sets have a correlation coefficient of > 0.90 indicating that for most genes, the model fits the data accurately.

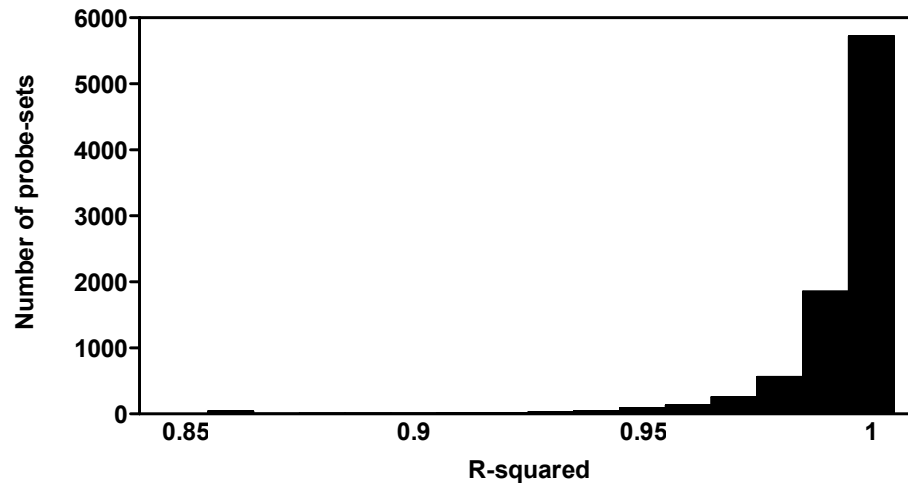


Figure 1. Distribution of correlation coefficients (R^2) between the signals calculated by the linear model and the actual signals from the perfect match (PM) probes.

Numbers of differentially expressed genes

Upon applying a FDR control-level at 0.005 according to Benjamini-Hochberg, MS was associated with up-regulation of 130 genes and down-regulation of 15 genes. An overview of the genes that demonstrate increased or decreased expression is given in table 2.

Sandwich ELISA

The sandwich ELISA was used to examine whether mechanical stress results in ssDNA breaks. Figure 2 demonstrates no difference in single-strandedness of DNA between stretched and control cells in both myocyte-enriched cultures and pure fibroblasts. The staurosporine-treated cells, however, had high levels of ssDNA, illustrating the sensitivity of the sandwich ELISA.

Table 2. Genes differentially expressed after 24 h of mechanical stress in cardiac cells.

LocusLink ^a	Representative Public ID ^b	Gene Name ^c	Fold Change ^d
<i>Adhesion/matrix</i>			
94339	AB010960	Matrix metalloproteinase 23	1.1
84032	M21354	collagen, type III, alpha 1	1.1
85250	AI179399	collagen, type V, alpha 2	1.2
116640	U09401/ U15550	tenascin C	1.5
24914	AI234060	lysyl oxidase	1.2
25615	M81687	syndecan 2	1.3
25473	AA900848	laminin, beta 2	1.3
140433	Y08882	laminin, alpha 5	1.3
25661	M28259/ U82612/	fibronectin 1	1.6
25181	AA859830	biglycan	1.6
25683	U57362	procollagen, type XII, alpha 1	1.3
<i>GF, interleukines and receptors</i>			
83785	L20913	vascular endothelial growth factor	1.4
25341	U94330	TNF receptor family, member 11b	1.4
25267	Z14118	PDGF receptor alpha polypeptide	1.4
24629	Z14119	PDGF receptor beta polypeptide	1.4
300438	X13722	low density lipoprotein receptor	1.3
24494	M98820	interleukin 1 beta	-1.3
25663	M95578/ U14010	interleukin 1 receptor, type I	1.3
83570	AF014827	c-fos induced GF (VEGF D)	1.3
59106	AF016901	latent TGF beta binding protein 2	1.3
25529	L19933	tyrosine phosphatase receptor type D	1.2
<i>cholesterol synthesis</i>			
83792	AF036761/ M15114/	stearoyl-Coenzyme A desaturase 2	1.3
29637	AI177004/ X52625	3-HMG-Coenzyme A synthase 1	1.2
<i>fatty acid and phospholipid synthesis</i>			
113976	AI236284/ D85189	fatty acid Coenzyme A ligase, long	1.3
50671	X13527	fatty acid synthase	1.5
29653	U36771	glycerol-3-phosphate acyltransferase	1.3
140544	AA925887	phosphate cytidyltransferase 1	1.2
114561	AA998446	phosphatidylinositol transfer protein	1.4
29554	Z12158	pyruvate dehydrogenase E1 alpha 1	1.2
24645	U20195	phosphoglucomutase 1	1.3
25739	M27726	brain glycogen phosphorylase	1.4
25058	J04526	hexokinase 1	1.3
24159	J05210	ATP citrate lyase	1.3
24533	M54926	lactate dehydrogenase M	1.1
<i>contractile filaments</i>			
60571	AI237258	MYB binding protein 1a	1.3
79433	U15766	myosin heavy chain 10, non-muscle	1.2
29556	AI104924	myosin heavy chain, polypeptide 6	1.2
25745	U15764	myosin, heavy polypeptide 9	1.3
24852	J02780	tropomyosin 4	1.4

LocusLink ^a	Representative Public ID ^b	Gene Name ^c	Fold Change ^d
286890	AA859305	tropomyosin isoform 6	1.1
65261	X74800	myosin Myr2 I heavy chain	1.4
<i>calcium homeostasis</i>			
64152	U39875	calcium binding protein p22	1.2
29716/ 171140	U31772/ M92905	calcium channel voltage-dep. L type	-1.1
	U73503	CaM kinase II gamma	-1.2
29144	L18889	calnexin	1.3
25403	Y13591	calpastatin	1.4
64202	X53363	calreticulin	1.2
29715	U04937	Na(+)-Ca2+ exchanger isoform 1	1.4
24246	AA894330/ L13406	calcium/calmodulin-dependent	1.3

a Locus Link number

b Genebank accession number

c Gene name and function

d Fold Change, up- or down regulation compared to the expression in untreated cells

Changes in expression of components of the extracellular matrix

MS of cardiac cells induced an increased expression of tenascin-C and procollagen XII. Several other extra cellular matrix (ECM) components that are controlled indirectly by MS showed also increased expression. For instance, syndecan-2, a protein that links the cytoskeleton to the ECM, and the extracellular proteoglycan biglycan showed increased expression. This phenomenon has been observed in infarcted myocardium of rats (Ahmed et al., 2003) and mice (Finsen et al., 2004). Lysyl oxidase, an extracellular copper-containing enzyme that initiates cross-linking of collagens and elastin, also demonstrated increased expression. Other genes that showed MS-induced up regulation include collagen III alpha 1, collagen V alpha 2, the laminins alpha 5 and beta 2, fibronectin and metalloproteinase 23, all proteins involved in keeping the balance between ECM production and degradation.

Growth factors and their receptors

MS of cardiac cells induced an increased expression of VEGF, PDGF-receptor (β polypeptide), PDGF-receptor (α polypeptide), latent TGF β -binding protein 2, IL-1-receptor (type I), TNF-receptor superfamily member 11b, and VEGF-D. These

findings support the view that the hypertrophic response induced by MS occurs, at least partly, via paracrine mechanisms (van Wamel et al., 2002).

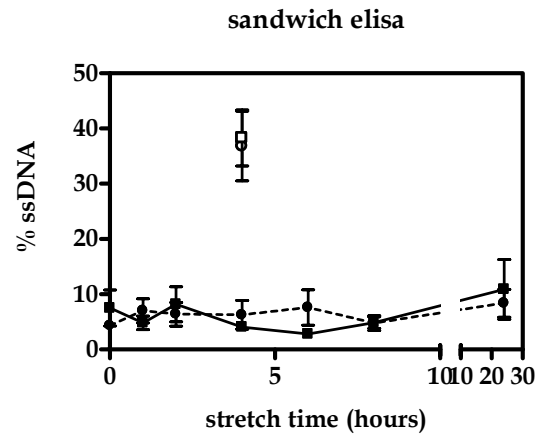


Figure 2. Percentage of single stranded DNA (ssDNA) in alkaline treated DNA from cells stretched for 0-24 h. The filled squares represent the percentage of ssDNA in myocyte-enriched cell-populations while the filled circles represent the percentage of ssDNA in the fibroblast cell-population. The open symbols ($\square$: myocyte-enriched cell-populations, $\circ$: fibroblast cell-population) show the induction of ssDNA after treatment of staurosporine for 4 hours, which was used as a positive control.

Energy metabolism

The present study presents evidence of MS-induced activation of glycolysis, as the genes encoding hexokinase-1, phosphoglucosmutase-1, brain glycogen phosphorylase and pyruvate dehydrogenase E1 α 1 are unregulated after 24 h of MS. Mitochondrial energy metabolism does not seem to be activated by MS. Interestingly the gene encoding extramitochondrial ATP-citrate lyase was unregulated by MS, which favors the removal of citrate from the tricarboxylic acid (Krebs) cycle to serve as a precursor of extramitochondrial acetyl-CoA for fatty acid biosynthesis. Several genes encoding proteins involved in fatty acid biosynthesis are unregulated, such as fatty acid synthase, fatty acid CoA ligase (long chain 4) and stearyl-CoA desaturase 2.

Fatty acids are, besides becoming oxidized in the mitochondria, used in the synthesis of phospholipids. The gene encoding phosphocholine cytidyltransferase (α -isoform) was found to be unregulated during MS. In addition, the gene encoding HMG-CoA synthase 1 is unregulated, leading to synthesis of HMG-CoA to be used for sterol synthesis and for ketone body formation (e.g. acetoacetate).

Our gene array study indicates that MS causes an up regulation of glycolysis and fatty acid biosynthesis, to be used for synthesis of phospholipids and sterols, like cholesterol. Phospholipids and cholesterol are the building blocks of plasma membranes, and their increased synthesis is compatible with MS-induced hypertrophic growth.

Contractile filaments

Results of the present study demonstrate that MS of cardiac cells induce an up-regulation of myosin heavy chain species, particularly non-muscle isoforms, and tropomyosins.

Calcium homeostasis

The present study did not demonstrate an increase in expression of SERCA2 and phospholamban in cardiac cells 24 h after MS. Maybe 24 h of MS marks the moment that increased expression turns to decreased expression. Two types of voltage dependent calcium channels, namely the N-type $\alpha 1$ beta and the L-type $\alpha 1D$ calcium channels were down-regulated and the Na^+/Ca^{2+} exchanger was up-regulated. One of the SR proteins that bind Ca^{2+} -ions within the SR, namely calreticulin, is up-regulated in the present study. However, gene expression of another Ca^{2+} -binding SR protein, calsequestrin, remained unchanged. A protein that is involved in calcium homeostasis and showed up-regulation in our study is calmodulin-dependent protein kinase-II (CaMPKII).

Validation of micro array results by semi-quantitative PCR

Gene expression changes detected using micro arrays were validated by semi-quantitative PCR, using RNA from CM-enriched cultures at 24 hours after MS. The increased gene expression of tenascin-C and biglycan were confirmed with PCR in two independent experiments. In these two experiments, tenascin-C gene expression increased 1.59 and 1.64 times, respectively. Biglycan gene expression increased 1.42 and 1.25 times, respectively. Figure 3 shows a representative result of the tenascin-C PCR products.

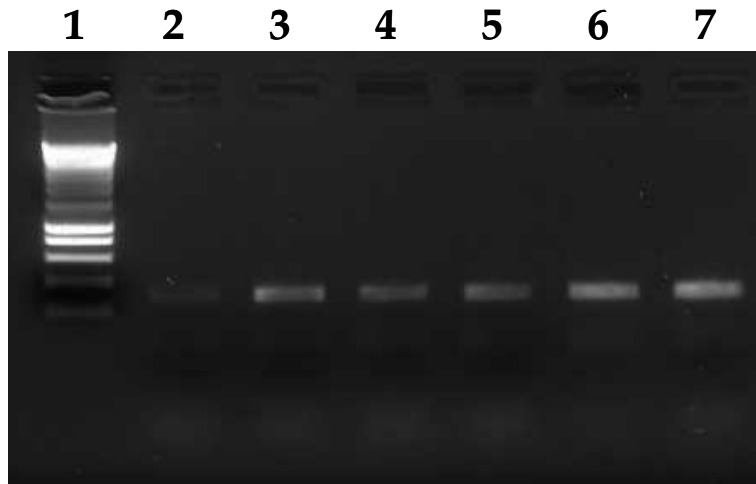


Figure 3. Representative result of the tenascin-C PCR. Total RNA was isolated from CM-enriched cultures at 24 hours after MS or control treatment. After cDNA synthesis, semi-quantitative PCR was used to determine tenascin-C gene expression. Lane 1: smart ladder; lane 2: negative control; lane 3: positive control; lanes 4 and 5: CM-enriched after control treatment; lanes 6 and 7: CM-enriched after MS.

Discussion

Mechanical stress plays an important role in the cardiac adaptation to hemodynamic overload. In addition to triggering the force-length mechanism and homeometric autoregulation, mechanical stress (MS) activates signaling mechanisms involved in hypertrophic growth of cardiac myocytes. The view that mechanical stress itself is the primary factor for hypertrophic response is supported by ex vivo experiments. In this study we tried to elucidate whether the changes in gene transcription due to mechanical stress in vitro could improve insights in the process leading to the initiation of the hypertrophic response. In the next paragraphs we will discuss our observations in relation to the changes observed in hypertrophic growth of the heart.

Changes in expression of extracellular matrix proteins

Cardiac hypertrophy is associated with fibroblast-mediated remodelling of the heart (Piper et al., 2003), which requires a rearrangement of cells and ECM to form a new geometry. Left ventricular volume and pressure overload cause expression

of different patterns of remodelling of the ECM, indicating that a feedback mechanism exists by which cells, that sense mechanical stress via ECM-integrin interactions, respond by a specific pattern of protein expression. The latter will lead to remodelling of ECM to meet changing mechanical requirements. Mechanical stress can regulate the production of ECM proteins indirectly, by stimulating the release of paracrine growth factors, or directly, by triggering an intracellular pathway that activates gene transcription. Whereas the expression of many ECM components is likely to be controlled indirectly by mechanical stress, there is evidence that the production of tenascin-C and collagen XII is under the direct control of mechanical stress (Chiquet et al., 1996; Chiquet, 1999; Chiquet et al., 2003). The response to a change in stretch is rapid and reversible, and is reflected on levels of mRNAs encoding these proteins. Both genes contain promoters having “stretch-responsive” enhancer regions, similar to “shear stress response elements”.

In normal heart, tenascin-C is only present during the early stages of development; however it reappears in pathological states (Schellings et al., 2004). The expression of tenascin-C is up regulated at a very early stage of myocarditis (Imanaka-Yoshida et al., 2002). During the acute phase after myocardial infarction, interstitial cells (mainly fibroblasts) in the border zone synthesize tenascin-C (Imanaka-Yoshida et al., 2001). Furthermore mechanical strain induces tenascin-C in cardiac myocytes through a NF- κ B-dependent and angiotensin II-independent mechanism (Yamamoto et al., 1999). Tenascins are “anti-adhesive” proteins that interfere with the attachment of cells with ECM substrates by changing the structure of focal adhesions and cytoskeletal filaments (Orend and Chiquet-Ehrismann, 2000). Thus tenascins may facilitate ventricular remodelling by loosening the cardiac myocytes from the ECM. The large number of genes encoding ECM components that were up regulated by MS in our study demonstrates that besides cardiac myocytes, the fibroblasts, which are the main producers of ECM components, are key players in the MS response. Apparently, the paracrine influences that cardiac myocytes have on fibroblasts (Manabe et al., 2002) is of utmost importance, as pure fibroblast cultures exposed to MS did not respond to MS by up regulation of these ECM-encoding genes.

Integrins and growth factors and their receptors

Important candidates for sensing mechanical stress are the integrins, transmembrane receptors that form the primary link between extracellular matrix

and cytoskeletal structures. Integrins are a complex family of non-covalently associated heterodimeric transmembrane receptors that are composed of alpha and beta subunits.

Apart from the numerous pathways the integrins themselves signal through, they also interact with, amongst others, growth factors (Ross, 2004). Between growth factors and integrins bi-directional cross-talk exists that appears to be subunit specific.

In the present study no changes in expression of genes encoding integrins were observed following 24 h of MS, however several genes encoding growth factors and growth factor receptors were found up regulated by MS.

Energy metabolism

In post infarction-remodeled hearts and in failing hearts, a shift of myocardial creatine kinase (CK) expression from M to B subunits has been reported (Ye et al., 2001). The CK system plays an important role in myocardial energy metabolism by maintaining ADP levels high at the mitochondria, where ATP is generated, and by maintaining ATP levels high at sites of ATP utilization. ATP is synthesized mainly in the mitochondria through oxidative phosphorylation and “transported” (via the creatine/creatine phosphate shuttle) to the contractile apparatus, where it is hydrolyzed by myosin-ATPase to generate force. In addition, cardiac overload causes a shift of lactate dehydrogenase (LDH) from the H (heart) to the M (muscle) subunit (Piper et al., 2002).

The results of the present study demonstrate that MS of cardiac cells induces an up-regulation of the LDH M subunit, but expression changes in M or B subunits of CK and the LDH H subunit have not been observed. Two explanations can be given: either the duration of MS was too short in our study, or other factors or conditions should be present as well, such as ischemia.

The shift from oxidative to glycolytic metabolism on a gene transcription level, as has been reported in hypertrophic animals (Kagaya et al., 1990), is observed in MS-treated cardiac myocytes. Moreover, fatty acid synthesis is stimulated by MS and lipids are routed to phospholipid synthesis to meet the expansion of membrane area in the growing cardiac myocytes.

Contractile filaments

The contractile machinery of the heart consists of proteins primarily concerned with contraction (actin and myosin) and proteins with a regulatory function (troponin and tropomyosin). In living muscle contraction will only take place in the presence of ATP and Ca^{2+} ions. Upon release of Ca^{2+} ions from the SR, intracellular Ca^{2+} ion concentration rises and contraction takes place. Relaxation is initiated by a rapid re-uptake of calcium ions by the calcium pump of the SR (SERCA).

Myosin consists of two polypeptide chains termed myosin heavy chains (MHC) and four polypeptide chains called myosin light chains (MLC). The site for ATPase activity is present in the globular head of the MHC, which also carries the actin binding site. Since there are two types of MHC, the α -MHC and the β -MHC, three types of myosin can be found, myosin containing two α -MHC (V1), or two β -MHC (V3), or the heterodimer containing one α -MHC and one β -MHC (V2). The specific ATPase activity of these three MHCs decreases in the order V1, V2 and V3 (Alpert and Mulieri, 1986).

In rodents, left ventricular overload causes a shift in expression from α -MHC to β -MHC expression, which is considered to represent reintroduction of the fetal gene program (Izumo et al., 1987; Iwanaga et al., 2000; Spangenburg et al., 2002; Stephens and Swynghedauw, 1990; Swynghedauw, 1999). This shift in expression is associated with a lower myosin ATPase activity and consequently a lower shortening velocity. This slowing improves efficiency of contraction (Alpert and Mulieri, 1982). Also in the human hypertrophied heart a down-regulation of α -MHC and an up-regulation of β -MHC were observed (Lowes et al., 1997; Nakao et al., 1997; Reiser et al., 2001). To our surprise the only myosin heavy chain genes that were found unregulated in cardiac myocytes after 24 h of MS were non-muscle subtypes, instead of muscle β -MHC.

Calcium homeostasis

Several genes that encode proteins involved in calcium homeostasis are down-regulated in hypertrophied hearts, although in the initial stages of cardiac overload these genes showed increased expression. Probably, the moment that increased expression turns to decreased expression marks the end of myocardial compensation to overload and indicates that myocardial failure sets in.

The expression of SERCA2 and its regulatory protein phospholamban are decreased after the onset of overload (Arai et al., 1993; Hashida et al., 1999; Matsui et al., 1995). As a result the intracellular calcium uptake and calcium release by SR are slowed down, which may explain the reduced velocity of relaxation and the reduced force generation, observed in hypertrophied hearts. As a compensatory response to the reduced calcium uptake of the SR, both mRNA and protein levels of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) are increased (Studer et al., 1997). In addition, the expression of the SR Ca^{2+} release channel, the ryanodine receptor (RyR), is also decreased (Brillantes et al., 1992; Naudin et al., 1991). These abnormalities in calcium handling leads to a decreased amplitude of the calcium transient (Gomez et al., 2001), a prolongation (Beuckelmann et al., 1992) of the calcium transient and an increased diastolic calcium concentration. The increased diastolic calcium concentration leads to activation of the heterotrimer calcineurin, a calcium dependent phosphatase. The biological effects of calcineurin, a potent initiator of a program of gene expression that leads to cardiac hypertrophy, are mediated through two classes of transcriptional effectors, nuclear factor of activated T-cells (NFAT) and myocyte enhancer factor 2 (MEF2) (Molkentin, 2004; Molkentin et al., 1998).

We found decreased expression of genes encoding two types of Ca^{2+} channels, up-regulation of SR Ca^{2+} binding protein gene, calreticulin and increased expression of CaMPKII. The increase in CaMPKII transcription that we found is interesting as CaMPKII is responsible for phosphorylation of proteins (such as phospholamban), leading to a compensatory increase of inotropy, chronotropy and lusitropy.

Apoptosis

Although apoptosis has been mentioned in relation to myocardial hypertrophy (Teiger et al., 1996), exposure of cardiac cells to MS for 24 h did not induce an increase in ssDNA fragments. In a previous study we demonstrated that MS for 24 h did not induce an increase in caspase-3 activity, nor fragmentation of [^3H]-thymidine prelabeled DNA in heart cell cultures (Persoon-Rotherth et al., 2002). Apparently, up regulation of genes encoding pro-apoptotic proteins is not part of the hypertrophic process per se, but rather belongs to the transition phase of hypertrophy to heart failure {Ding, Price, et al. 2000 57 /id}. Myocardium of failing hearts showed increased incidence of apoptotic cells (Narula et al., 1996; Olivetti et al., 1997), and one of the factors that may be responsible is the increased

concentration of angiotensin-II that has potent pro-apoptotic effects (Cigola et al., 1997; Kajstura et al., 1997).

Conclusion and limitations

In summary, the changes in gene expression described above, strengthen the view that MS is one of the initiation factors of the hypertrophic response observed in overloaded myocardium. In this study the changes in gene expression that occurred in pure fibroblast cell-populations were examined and compared to the changes that occurred in mixed myocyte-fibroblast cell-populations. Upon applying the FDR according to Benjamini-Hochberg 15 out of 145 genes that were differentially expressed, appeared to be cell-type specific. However since the mixed cell population consists of both cardiac myocytes and fibroblasts it is unclear whether the observed changes in gene expression are the result of changes in gene expression in cardiac myocytes or whether they are caused by paracrine interactions between the two cell types. Therefore it would be interesting to examine the response of pure myocyte cell populations to MS. Future studies need to put effort in purification of cardiac myocytes and investigation of their response to MS.

Acknowledgements

This study was financially supported by the Netherlands Heart Foundation (grant no. 97.176), the Interuniversity Research Institute of Radiopathology and Radiation Protection (grant no. 9.0.13) and the European Union. The authors would like to thank Rebecca Esveltdt-Van Lange and Cindy Schutte-Bart for skilful technical support and Mr. P. de Jong of the Animal Care Facilities.

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**Microarray analysis of gene expression
profiles of cardiac myocytes and fibroblasts
after mechanical stress, ionising or
ultraviolet radiation.**

Microarray analysis of gene expression profiles of cardiac myocytes and fibroblasts after mechanical stress, ionising or ultraviolet radiation.

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Abstract

Background During excessive pressure or volume overload, cardiac cells are subjected to increased mechanical stress (MS). We set out to investigate how the stress response of cardiac cells to MS can be compared to genotoxic stresses induced by DNA damaging agents. We chose for this purpose to use ionising radiation (IR), which during mediastinal radiotherapy can result in cardiac tissue remodelling and diminished heart function, and ultraviolet radiation (UV) that in contrast to IR induces high concentrations of DNA replication- and transcription-blocking lesions.

Results Cultures enriched for neonatal rat cardiac myocytes (CM) or fibroblasts were subjected to any one of the three stressors. Affymetrix microarrays, analysed with Linear Modelling on Probe Level, were used to determine gene expression patterns at 24 hours after (the start of) treatment. The numbers of differentially expressed genes after UV were considerably higher than after IR or MS. Remarkably, after all three stressors the predominant gene expression response in CM-enriched fractions was upregulation, while in fibroblasts genes were more frequently down-regulated. To investigate the activation or repression of specific cellular pathways, genes present on the array were assigned to 25 groups, based on their biological function. As an example, in the group of cholesterol biosynthesis a significant proportion of genes was up-regulated in CM-enriched fractions after MS, but down-regulated after IR or UV.

Conclusion Gene expression responses after the types of cellular stress investigated (MS, IR or UV) have a high stressor and cell type specificity.

Background

The mammalian myocardium contains several cell types, of which the cardiac myocytes (CM) make up most of the heart's mass. Although a small proportion of CM in the adult myocardium remains mitotic, most CM lose the capacity to undergo cell division shortly after birth (Tamamori-Adachi et al., 2003). In the adult heart, approximately 70% of the cells is represented by non-myocytes, most of which belong to the fibroblast compartment. In this study, we investigate how the stress response of cardiac cells to increased mechanical stress (MS) can be

compared to genotoxic stresses induced by two DNA damaging agents, ionising radiation (IR) and ultraviolet radiation (UV).

In cardiac cells, MS is increased during excessive pressure or volume load of the heart, as seen in hypertensive and valvular heart disease. This results in an adaptive growth response leading to structural and functional cardiac changes, including CM hypertrophy and hyperplasia of fibroblasts, to compensate for the increased workload (Brilla and Maisch, 1994). In vitro cyclic stretch of rat cardiac cells has been shown to be an appropriate model for cellular changes that occur during overload of cardiac muscle in vivo (Sadoshima et al., 1992). Mechanical signals may be transferred to the nucleus of cells through integrin receptors, cytoskeletal filaments and nuclear scaffolds (Alenghat and Ingber, 2002) and through ion channels, ion exchangers and hormone receptors (Ruwhof and van der Laarse, 2000).

Radiation induced heart disease (RIHD) has been recognised as a late adverse effect of thoracic radiotherapy if the heart was situated in the radiation field (Adams et al., 2003). IR induces the formation of reactive oxygen species that react with different components of the cell, thereby inducing macromolecular lesions. IR can activate several signal transduction pathways, involving growth factor receptors, death receptors and DNA damage sensing proteins (Dent et al., 2003). Primary fibroblasts in culture are known to go into senescence shortly after IR (Di Leonardo et al., 1994), after which terminal differentiation of these cells is induced (Rodemann et al., 1991). Cultures of CM do not demonstrate cell death, nor a loss of function upon a single dose of 10,000 rad (~100 Gy) (Lampidis et al., 1975; Petrovic et al., 1977).

The density of DNA damaging events after UVC, which include helix-distorting photolesions, is three orders of magnitude higher than the density of DNA damage that occurs after IR (Ward, 1994) and sufficient to block cellular DNA replication and transcription. Signal transduction after UV is mediated via components of the cellular membrane, involving growth factor receptors, and via DNA damage sensing proteins (Dent et al., 2003). UV is known to induce cell cycle arrest and apoptosis in several cell types (Kulms and Schwarz, 2002; van der Wees et al., 2003).

The aim of the study was to identify and compare differentially expressed genes (upregulated or down-regulated when compared with untreated controls) in cardiac cells in response to MS, IR or UV. To this purpose, cultures enriched for ventricular CM or fibroblasts were exposed to one of the three stressors. Differentially expressed genes were identified using Affymetrix GeneChips.

Several statistical methods have been developed to analyse Affymetrix gene expression microarrays. We used a method based on Linear Modelling on Probe Level (Chu et al., 2002) to describe the signal of every perfect match (PM) probe. Because overall changes in the expression of functionally related genes are more informative than the expression pattern of single genes, genes in microarray studies can be assigned to functional groups (Jen and Cheung, 2003). In the present study, such an approach was used to classify genes, based on biological function or on the role of a gene product in common intracellular pathways.

Methods

Cell culturing

The experiments were performed with permission of the local committee on animal experiments, installed by the University of Leiden according to the Dutch law. Cardiac cells were isolated from ventricles of neonatal rat hearts, and cultured as described before (Van der Laarse et al., 1979; van der Wees et al., 2003). By applying a pre-plating method, cultures enriched for CM and cultures of fibroblasts were obtained. After 2 days of culturing with 5% horse serum (HS), culture media of CM-enriched cultures were replaced by media containing 2.5% HS. One day later, these cultures were subjected to UV, IR or MS. Three days after isolation, cultures of fibroblasts were subcultured for 6 days in medium containing 10% foetal bovine serum (FBS). Then, culture medium was replaced by medium containing 2.5% FBS. One day later, confluent cell cultures were subjected to UV, IR or MS.

Irradiation and cyclic stretch

Cells were subjected to a single dose of 8.5 Gy of X-rays at room temperature, using a 6 MV accelerator (SL 75-5 Philips), operated at a dose rate of 8 Gy min⁻¹. During irradiation, sham-irradiated control cells were kept at room temperature. After irradiation, cell cultures were maintained at 37 °C for 24 h. In another experiment cells were irradiated with 10 J/m² UVC at room temperature at a dose rate of 0.2 J/m² per s. Before irradiation, medium was collected and cells were rinsed with PBS. Following irradiation, growth medium was returned to the cultures and the cells were incubated for an additional 24 h. Control cells received

a similar treatment except for UV irradiation. To subject cells to cyclic stretch, CM-enriched cells ($54\pm5\%$ CM) and fibroblasts ($95\pm2\%$ non-myocytes) were cultured in 6-well Flex I culture plates (Flexcell, Hillsborough, NC, USA), coated with collagen I. Medium was changed to medium containing 2.5% serum and after 24 h plates were placed in the Flexercell Strain Unit FX-2000® (Flexcell) in which the frequency and magnitude of stretch were regulated by a computer-controlled vacuum pump. The apparatus applied an equiaxial cyclic stretch of 20% elongation to the wells at a frequency of 60 cycles/min (1 Hz). Stretch was applied for 24 h. Control cells were grown in identical culture plates and incubated in the same incubator as the stretched cultures, but were not mounted in the Flexercell Strain Unit.

Cell harvesting after irradiation

Twenty-four hours after IR or UV, non-irradiated and irradiated CM-enriched cultures were trypsinised and subjected to centrifugal elutriation (Boerma et al., 2002b). The proportion of CM in each elutriation fraction was determined by flow cytometric analysis of myosin expression, as described before (Boerma et al., 2002b). After elutriation of IR-exposed cultures, CM-enriched fractions contained $74\pm3\%$ CM and after elutriation of UV-exposed cultures, CM-enriched fractions contained $85\pm3\%$ CM. Because of the high purity of the fibroblast cultures ($95\pm2\%$ non-myocytes), no centrifugal elutriation was applied on these cells. CM-enriched fractions and cultures of fibroblasts were used for RNA isolation.

Cell harvesting after MS

After undergoing MS or control treatment for 24 h, cells were collected from CM-enriched cultures and from cultures of fibroblasts by trypsinisation. The proportion of CM in the cell cultures was determined by flow cytometric analysis of myosin expression as described before (Boerma et al., 2002b). CM-enriched cell cultures, containing $54\pm5\%$ CM, and fibroblast cultures, containing $95\pm2\%$ non-myocytes, were used for RNA isolation.

RNA isolation and labelling

RNA was isolated applying an RNeasy® kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Ten to 14 µg of total RNA from CM enriched fractions and 7.5 to 14 µg of total RNA from fibroblast cultures was used for labelling. Per experiment, the quantities of total RNA from irradiated and control cells were equal. cDNA was synthesised using the Gibco BRL Superscript system (Invitrogen, Carlsbad CA, USA). Briefly, single stranded cDNA was synthesized using Superscript II reverse transcriptase and T7-oligo(dT)24 primers at 42°C for 1 h. Double stranded cDNA was obtained by using DNA ligase, DNA polymerase I and RNase H at 16°C for 2 h, followed by T4 DNA polymerase at 16°C for 5 min. After clean up with a phase lock gel (Qiagen), ds-cDNA was used for in vitro transcription (IVT). cDNA was transcribed using the BioArray HighYield ® RNA transcript labelling kit (Enzo Lifesciences, Pharmingdale NY, USA) in the presence of biotinlabelled ribonucleotides, or using the MEGAScript T7® kit (Ambion, Austin TX, USA), in the presence of biotin-labelled CTP and UTP. In each experiment, cDNAs from control cells and stressed cells were labelled simultaneously using the same labelling kit. After clean up with a RNeasy kit (Qiagen), the biotin-labelled IVT-RNA was fragmented in a buffer containing 40 mM Tris-acetate (pH 8.1), 100 mM potassium-acetate and 30 mM magnesium-acetate, at 94°C for 35 min.

Hybridisation of IVT-RNA

RG-U34A arrays (Affymetrix, Santa Clara CA, USA) were used, representing ~7000 transcripts, of which 6399 had a unique LocusLink identification number (ID) at the time of data analysis. Biotin-labelled IVT-RNA was hybridised to the arrays at 45°C for 16 h according to the manufacturer's instructions. After hybridisation, the arrays were washed in a GeneChip Fluidics Station 400 with a non-stringent wash buffer at 25°C followed by a stringent wash buffer at 50°C. After washing, the arrays were stained with a streptavidin-phycoerythrin complex. After staining, intensities were determined with a GeneChip scanner, controlled by GeneChip software (Affymetrix). The intensities were background corrected using gcrma (Wu and Irizarry, 2004) and normalized at the probe level by VSN (Huber et al., 2002).

Data analysis

For each stressor, three independent experiments were performed. To describe the signal of every PM probe, the following linear model was used: $\text{signal} \sim P + T + E + TE$, based on a method described before (Chu et al., 2002). In our model, the symbols P, T, E and TE represent the effects of probe, treatment, experiment per stressor (1, 2, or 3) and the interaction between treatment and experiment, respectively. Subsequently, analysis of variance was applied on the treatment effect to determine the p-value for each probe-set. The p-values were corrected for multiple testing using the Benjamini Hochberg step-up procedure (Benjamini and Hochberg, 2004) which yielded q-values for the false discovery rates (FDR). The FDR level of control was set to 0.005, equivalent to selecting genes with a q-value < 0.005 (keeping up- and down-regulated genes separate by the sign of the treatment coefficient). Some genes are represented by more than one probe-set. A gene was determined to be differentially expressed when at least 50% of the probesets representing this gene showed significant up-regulation or down-regulation. To determine significant proportions of differentially expressed genes within functional groups, the hypergeometric probability P was calculated. $P < 0.005$ was considered significant. To determine the accuracy of the linear model that was used to describe PM signals, R^2 was calculated for every probe-set, using the following standard formula: $R^2 = 1 - \sum R_i^2 / \sum (\text{signal}_i - \text{mean signal})^2$, where R_i = observed-fitted value for signal_i and mean signal = the mean of observed signal_i . To compare data on differentially expressed genes with microarray data in literature on species other than rat, RESOURCERER of the Institute of Genomic Research (2004) was used.

RNA isolation and semi-quantitative PCR

Total RNA isolation, cDNA synthesis and semi-quantitative PCR were performed as described before (Boerma et al., 2002a). In short, total RNA was isolated using an RNeasy® kit (Qiagen). Following DNase (Amersham Pharmacia Biotech, Uppsala, Sweden) treatment, cDNA was synthesised using M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA) and oligo(dT) primers (Amersham). Semiquantitative PCR was performed, using the following primers and annealing temperatures: Tenascin C sense: CGA CAG TTT TGT TAT CAG GAT CAG, Tenascin C antisense: GGC ACA TAA GTA ATC CGG AAA T, 60 °C; Biglycan sense: CAA CAA CCC TGT GCC CTA CT, Biglycan antisense: GGT GT

GCT TCT TTG CTT CC, 65 °C. A competitive PCR was performed on GAPDH to correct for differences in cDNA concentrations. After agarose gel electrophoresis, intensities of PCR product bands were determined by Scion image analysis software (Scion Corporation, Frederick, MD, USA).

Results

Accuracy of the linear model

As an example for the accuracy of the linear model that was used to describe the PM probe signals, Figure 1A shows a dot plot of all PM probe signals as calculated by the linear model, against the actual PM probe signals determined from fibroblasts after UV. These data were used to calculate correlation coefficients (R^2) between the signals calculated by the model and the actual PM signals obtained. Figure 1B shows the distribution of R^2 values for fibroblasts after UV. The majority of probe-sets have a correlation coefficient ≥ 0.90 , indicating that the model used fitted the data accurately.

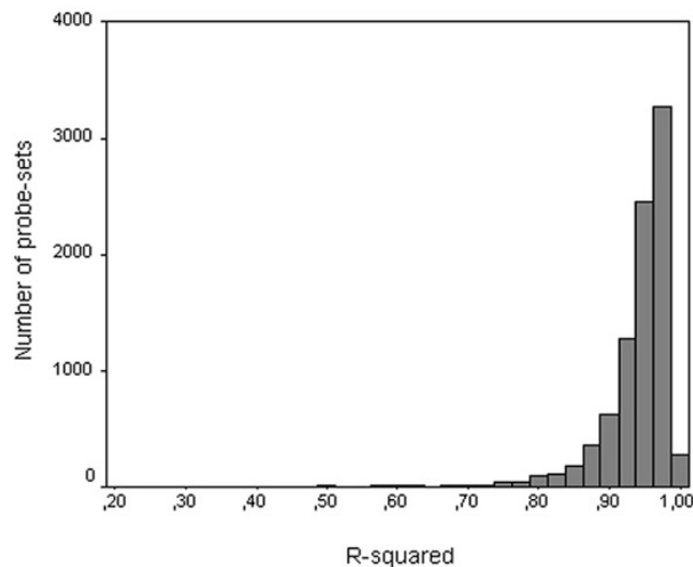


Figure 1 - Accuracy of the linear model. Dot plot of all PM probe signals as calculated by the linear model, against actual PM probe signals determined from fibroblasts after UV (A). The data presented in Figure 1A were used to calculate correlation coefficients (R^2) between the signals calculated by the linear model and the actual PM signals of fibroblasts after UV (B).

Numbers of differentially expressed genes

A Series entry (accession number GSE2032) at Gene Expression Omnibus (GEO), a public gene expression database of NCBI (2004), gives access to all microarray data generated in this study. Figure 2 represents the numbers of genes with a unique LocusLink ID that were up-regulated or down-regulated ($q < 0.005$) in CM-enriched cultures/fractions and cultures of fibroblasts after one of the three stressors. When using these criteria, the numbers of differentially expressed genes (up-regulated or down-regulated) after UV were considerably higher than after IR or MS. After each of the three stressors more genes were up-regulated in CM-enriched cultures/fractions than in cultures of fibroblasts. Conversely, higher numbers of down-regulated genes were determined in fibroblasts. These differences were most pronounced after MS (Figure 2A). Based on information available at NetAffxTM (2004), extended with standard textbooks and recent literature, genes with a unique LocusLink ID were assigned to several functional groups. Subsequently, the status of each gene within a functional group was determined in both cell populations (CM-enriched cultures/fractions and cultures of fibroblasts). Individual probe-sets within these functional groups and their q-value after the three stressors are listed in table 1 (see additional file 1).

In this table, downregulated genes are distinguished from up-regulated genes by a minus-sign in front of their q-value. Figure 3 shows the percentage of genes in a functional group that are differentially expressed after MS, IR or UV. In accordance with Figure 2, the highest percentages of genes were differentially expressed after UV (on average 39.4% for both cell populations), followed by IR (13.0%). In general, the percentages of differentially expressed genes were lowest after MS (8.3%). Several functional groups, including heat shock proteins and genes involved in cholesterol biosynthesis, showed high proportions of differentially expressed genes with a hypergeometric probability $P < 0.005$. These functional groups were considered to have significantly high percentages of differentially expressed genes. On the other hand, in the group of genes encoding for ion channels and exchangers, low percentages of differentially expressed genes with a hypergeometric probability $P < 0.005$ were determined, both after IR and UV.

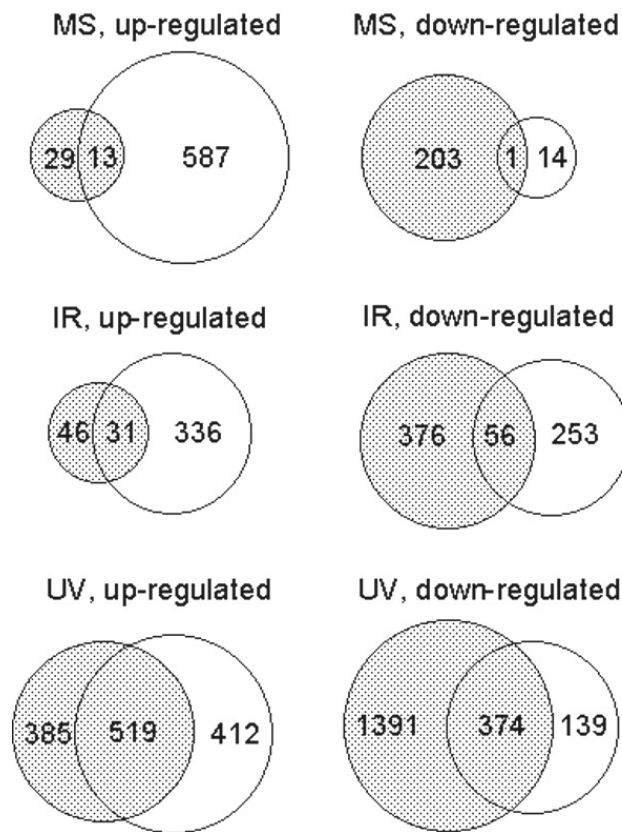


Figure 2 - Numbers of differentially expressed genes. Numbers of differentially expressed genes ($q < 0.005$) with a unique LocusLink ID in cultures of fibroblasts (dotted) and CM-enriched cultures after MS (A), in cultures of fibroblasts (dotted) and CM-enriched fractions after IR (B), or in cultures of fibroblasts (dotted) and CM-enriched fractions after UV (C). Overlapping parts of the circles represent genes that show differential expression both in CM-enriched cultures/fractions and cultures of fibroblasts.

Percentages of up-regulated genes

Figure 4 shows the percentages of differentially expressed genes that were upregulated after IR or after UV. In several functional groups, including p53 target genes and genes involved in mitosis, a significant percentage of differentially expressed genes was up-regulated (hypergeometric probability $P < 0.005$). Other functional groups, including cytoskeletal components and genes involved in cholesterol biosynthesis mainly had down-regulated genes. In all 25 functional groups, the hypergeometric probability P of the percentage of up-regulated genes after MS was not below the pre-set threshold of 0.005. Therefore, no significant

percentages of up-regulated genes were determined in any of the functional groups after MS (data not shown).

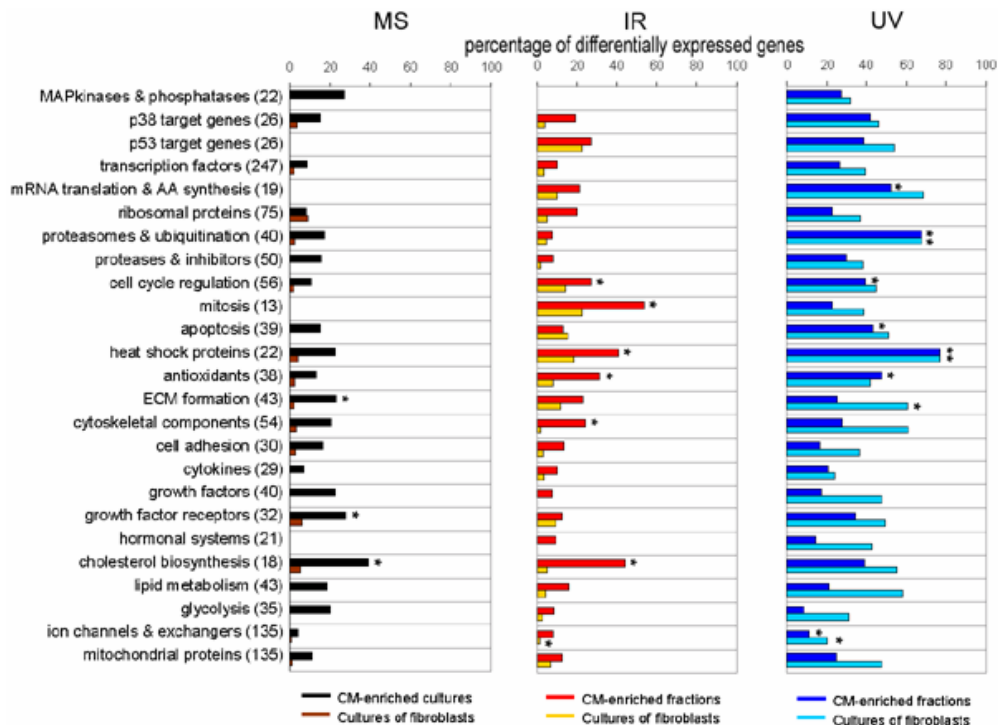


Figure 3 - Percentages of differentially expressed genes. Percentage of total number of genes within functional groups that are differentially expressed in CM-enriched cultures/fractions and cultures of fibroblasts after MS, IR or UV. Numbers between brackets represent total numbers of genes within a functional group. For example, of the 22 MAPKs and phosphatases found to be represented by the array, 27% were differentially expressed in CM-enriched fractions after UV. AA: amino acid. *Hypergeometric probability $P < 0.00$

General stress response genes

Probe-sets that showed an up-regulation or down-regulation after more than one stressor are listed in table 2 (see additional file 2). The overlap in responsive genes between IR and UV was larger than the overlap between MS and one of the radiation types. Both after IR and UV, several genes that are known to play a central role in the radiation response of cells, including p21, GADD153 and mdm2, were up-regulated. These genes were not up-regulated after MS. A striking large proportion of genes encoding cytoskeletal components were down-regulated both after IR and UV.

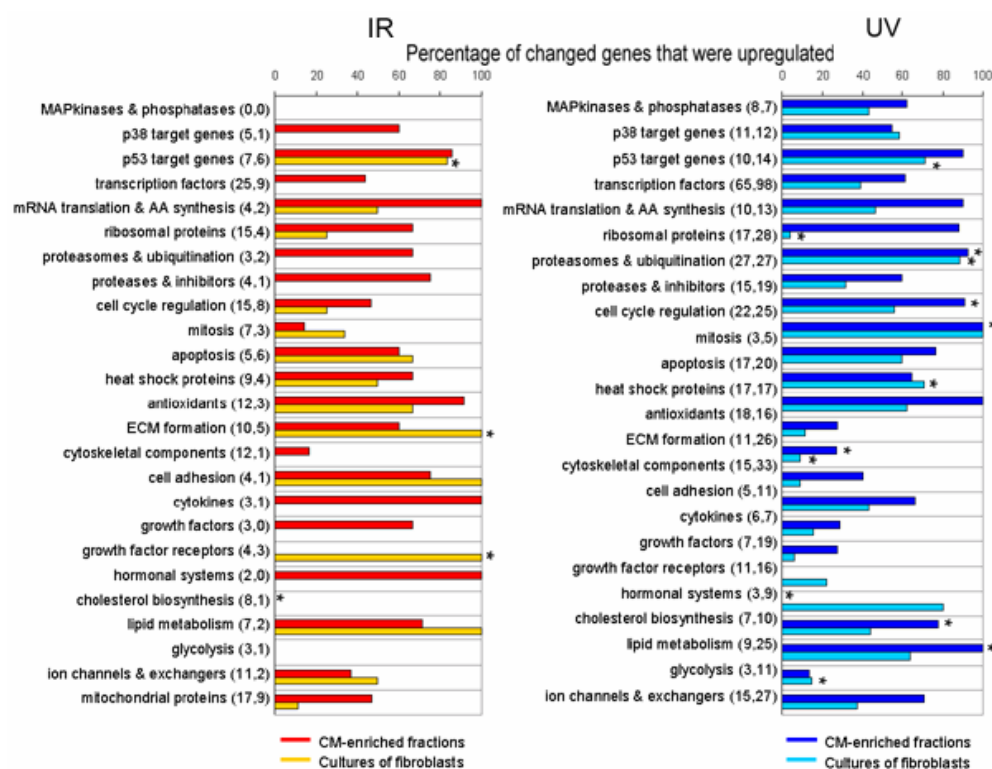


Figure 4 - Percentages of up-regulated genes. Percentage of changed genes that were up-regulated per functional group after IR or UV. Numbers between brackets represent numbers of differentially expressed genes in CM-enriched fractions and cultures of fibroblasts, respectively. For example, of the 8 MAPKinasases and phosphatases that were differentially expressed in CM-enriched fractions after UV, 63% were up-regulated. *Hypergeometric probability $P < 0.005$

Validation of microarray results by semi-quantitative PCR

Gene expression changes detected using microarrays were validated by semiquantitative PCR, using RNA from CM-enriched cultures at 24 hours after MS. The increased gene expression of Tenascin C and biglycan were confirmed with PCR in two independent experiments. In these two experiments, Tenascin C gene expression increased 1.59 and 1.64 times, respectively. Biglycan gene expression increased 1.42 and 1.25 times, respectively. Figure 5 shows a representative result of the Tenascin C PCR.

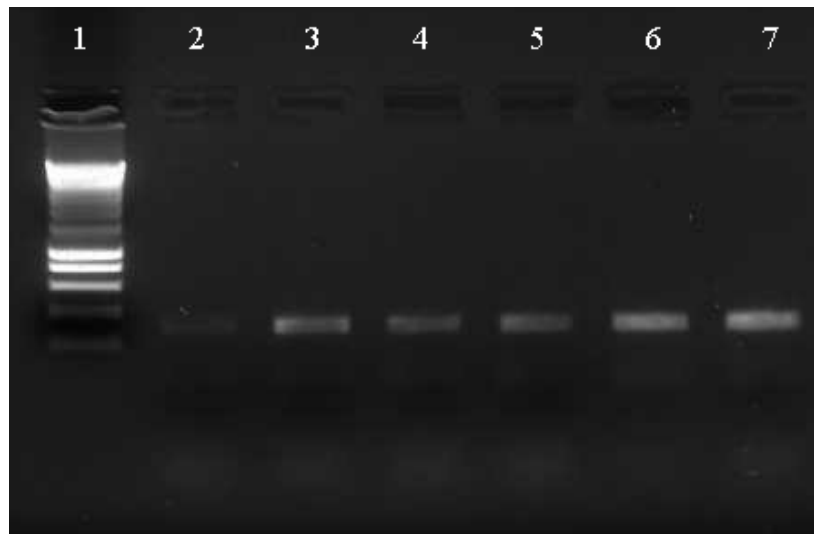


Figure 5 - Representative result of the Tenascin C PCR Total RNA was isolated from CM-enriched cultures at 24 hours after MS or control treatment. After cDNA synthesis, semi-quantitative PCR was used to determine Tenascin C gene expression. Lane 1: smart ladder; lane 2: negative control; lane 3: positive control; lanes 4 and 5: CM-enriched after control treatment; lanes 6 and 7: CM-enriched after MS.

Discussion

In this study, a linear model was used to describe GeneChip PM probe signals and to determine effects of three types of stressors on gene expression in two neonatal rat heart cell populations, consisting of CM that are known to be terminally differentiated cells and fibroblasts that still have the capacity to undergo mitosis. Several studies have shown a good correlation between Affymetrix GeneChip data and RT-PCR (Jen and Cheung, 2003) or Northern-blot (Bodyak et al., 2002; Sesto et al., 2002). In a previous study neonatal rat CM-enriched cultures and cultures of neonatal rat cardiac fibroblasts were irradiated with a single dose of 8.5 Gy and some mRNA transcripts were quantified by competitive PCR (Boerma et al., 2002a). In accordance with the present study, no significant changes in gene expression of transforming growth factor- β 1 (TGF- β 1), fibroblast growth factor-2 (FGF-2) and collagen type I were determined at 24 h after IR in cultures of cardiac fibroblasts. Moreover, no significant IR-induced changes were determined in gene expression of atrial natriuretic peptide (ANP) in CM-enriched cell populations in both the latter and the present study. On the other hand, CM-enriched cultures showed a reduced TGF- β 1 expression (by PCR) at 24 h after 8.5 Gy, which was not

observed in the present study. This might be due to differences in experimental design between the two studies, as in the former study CM-enriched cultures were obtained by incubation with bromodeoxyuridine to prevent fibroblast proliferation. Also, during and after irradiation, the cells were incubated in higher serum concentration than used in the present study. Here, differences between the two cell populations are observed in the predominant type of gene expression response, i.e. up-regulation versus down-regulation. After all three stressors, differentially expressed genes were mostly up-regulated ($q < 0.005$) in CM-enriched fractions, while in cultures of fibroblasts the majority of changed genes were down-regulated. After MS, these differences were most pronounced. Paracrine signalling is involved in the response of CM and cardiac fibroblasts in co-cultures subjected to MS (Ruwhof et al., 2001). In the CM-enriched cultures used in this study, remaining fibroblasts might stimulate gene transcription of the CM, resulting in higher numbers of up-regulated genes. It cannot be excluded that differences in level of toxicity between the three stressors applied in this study caused differences in gene expression levels.

Of the three stressors examined in this study, both IR and UV are known to induce oxidative stress. Accordingly, both stressors induced high numbers of up-regulated genes involved in anti-oxidative processes. The high percentages of up-regulated genes encoding heat shock proteins in both cell populations after IR and UV might indicate oxidative stress induced protein damage in these cells. In comparison, after MS only few genes encoding heat shock proteins were up-regulated in either cell population. Heinloth et al. (2003) proposed a model for the regulation of several gene expression patterns after IR and UV in human dermal fibroblasts, based on microarray analysis. The general view that p53 plays a central role in signal transduction after IR and UV was confirmed in their study. Moreover, IR down-regulated the expression of genes involved in mitosis. UV, on the other hand, induced the expression of genes involved in protein degradation and the MAPK pathway (Heinloth et al., 2003). The latter data are in accordance with the data of the present study, showing that UV did affect genes participating in the MAPK pathway (although not significantly) in both cell populations, but IR did not. Moreover, in CM-enriched fractions, IR resulted in a down-regulation of a large percentage of genes involved in mitosis. Several genes, involved in cell cycle regulation and mitosis are expressed in foetal cardiac myocytes and down-regulated in adult myocytes, in accordance with their terminal differentiation (Brooks et al., 1997; Garcia et al., 2002). Due to the close relation between foetal and neonatal cells, an expression of mitotic genes in the neonatal CM used in this study

can be expected. The high numbers of up-regulated genes involved in ubiquitination and protein degradation in both cell populations after UV are also in accordance with the study of Heinloth et al. (2003) and with other studies on cultured cells after UV (Sesto et al., 2002). These results might reflect the need of cells to replace molecules that were damaged by UV irradiation, although the proteasome is also proposed to play a role in several cellular processes, including DNA-repair, cell cycle regulation and cell survival after irradiation (McBride et al., 2003).

In CM-enriched cultures, MS led to a relatively high proportion of up-regulated genes that are involved in cholesterol biosynthesis. Among these genes, expression of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, which forms the starting enzyme of the mevalonate pathway and is considered to be the key enzyme in cholesterol biosynthesis, was up-regulated. This suggests that in neonatal rat cardiac myocytes cholesterol biosynthesis is stimulated after MS. Neonatal rat heart myocytes that undergo hypertrophy in culture show an increased biosynthesis and intracellular accumulation of cholesterol (Shmeeda et al., 1994). Moreover, the mevalonate pathway has been proposed to play a role in Ras activation in neonatal rat cardiac myocytes subjected to MS in culture, leading to hypertrophy (Kashiwagi et al., 1998). In vivo hypertrophy of the heart is also associated with elevated myocardial cholesterol contents (Okumura et al., 1992). An increased cholesterol biosynthesis in cardiac myocytes that undergo MS might therefore accompany a hypertrophic response of these cells. However, of the three foetal genes that are known to be re-expressed in hypertrophic myocytes, i.e. smooth muscle alpha-actin, ANP and beta-myosin heavy chain, only the last gene was up-regulated at the time point of investigation. In contrast to MS, IR and UV led to a down-regulation of genes involved in cholesterol biosynthesis in CM-enriched fractions. In previous studies, alterations in cholesterol contents of cardiac myocytes and fibroblasts were associated with alterations in protein to DNA ratios, levels of several enzymes including ATPases and phosphatases, and diffusion rates of membrane proteins (Bastiaanse et al., 1997; Yechiel et al., 1985; Yechiel et al., 1986). Moreover, a decrease in beating rate observed in aging cultures of CM was opposed by increased membrane amounts of cholesterol (Yechiel and Barenholz, 1985). The role of cholesterol biosynthesis in the cellular functions of cardiac cells after MS, IR or UV needs further investigation. Higher numbers of differentially expressed genes involved in ECM formation, including genes encoding collagens, fibronectin and laminin, were determined in CM-enriched cultures after MS than in cultures of fibroblasts. As mentioned before, paracrine signalling between CM and

remaining fibroblasts might play a role in cellular responses in these CM-enriched cultures. Alterations in expression of genes involved in ECM formation might originate from remaining fibroblasts after stimulation by CM. Interestingly, MS does not affect the expression of these genes in cultures of fibroblasts, which suggests that paracrine signalling from CM is necessary for alterations in expression profiles of genes involved in ECM formation in fibroblasts. The down-regulated genes encoding cytoskeletal components in cultures of fibroblasts after IR and UV, including myosin and troponin, are mostly CM-specific. Therefore, these genes are likely to originate from remaining CM in the fibroblast cultures, although their numbers were low (3-5%). The extremely low numbers of differentially expressed genes encoding for ion channels and exchangers might be explained by a low number of cardiac cell type-specific genes within this functional group.

Conclusions

MS, IR and UV mainly induce stress-specific and cell-type specific gene expression profiles in neonatal rat CM-enriched cultures and cultures of neonatal rat heart fibroblasts. Functional groups that show significant percentages of differentially expressed genes suggest that certain cellular pathways are activated after one or more stresses.

Additional material

Additional file 1

q-values of genes within functional groups Table 1. Excel-file containing the 25 functional groups of genes used in this study. Of every gene, Affymetrix probe-set IDs and q-values after the three types of stress are listed. Down-regulated genes are represented by a minus-sign in front of their qvalue. q-values<0.005 are marked red (in case of up-regulation) and green (in case of down-regulation).

[<http://www.biomedcentral.com/imedia/2018541103525243/sup1.xls>]

Additional file 2

General stress response genes Table 2. Excel file containing Affymetrix probe-set IDs, LocusLink ID and gene description of all probe-sets that showed an up-regulation or down-regulation after two or more stresses.

[<http://www.biomedcentral.com/imedia/1766719520525243/sup2.xls>]

Acknowledgements

This study was financially supported by the Netherlands Heart Foundation (grant no. 97.176), the Interuniversity Research Institute of Radiopathology and Radiation Protection (grant no. 9.0.13) and the European Union. The authors would like to thank Rebecca Esveldt-Van Lange, Cindy Schutte-Bart and Minka Bax for skilful technical support.

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8

Summary

The heart is built for life-long uninterrupted function, supporting blood flow through the organism. During life, the organism as well as its organs will become challenged by exogenous and endogenous stresses that should be coped with. To be able to adapt to stresses such as altered loading conditions, changes in myocardial perfusion and exposure to toxic agents, the heart possesses ample capabilities.

This thesis deals with the question how cardiac cells react to different stresses in an attempt to adapt to the new circumstances. In chapter two, six and seven we examined and described the effect of mechanical stress on cardiac cells. The chapters four and seven deal with the reaction of cardiac cells to DNA damage caused by either ionizing radiation or UV-irradiation. The last stress imposed to cardiac cells that was investigated, is integrin stimulation. The results of this study are restored in chapter three.

The normal function of the heart and the various cell types present in the organ are described briefly in chapter one. The cells used for the studies described in this thesis are the large cardiac myocytes, that make up most of the heart's mass and are responsible for the contractile function, and the fibroblasts, that contribute to $\approx 50\%$ of the total cell number and produce the extracellular matrix that contributes to the heart's tensile strength and stiffness.

Chronic overload of the heart that can be the consequence of high blood pressure or mitral valve insufficiency, induces a compensatory growth of the heart, termed hypertrophy. Although this hypertrophy is initially very beneficial, since it reduces wall stress, it can evolve into heart failure whenever overload sustains and the adaptation and functional alterations become insufficient. On the cellular level cardiac hypertrophy refers to supranormal growth of cardiac myocytes and hyperplasia of fibroblasts. During development of cardiac hypertrophy specific changes have been observed in both cell types, which are described briefly in chapter one.

Evidence accumulates that the response to mechanical overload includes apoptosis of cardiac myocytes. A decrease in the number of myocytes may contribute to impaired contractility of the whole heart, but whether this is the primary cause or the result of the progressive cardiovascular dysfunction remains unclear. To answer this question we investigated the direct effect of mechanical overload on the induction of apoptosis in cardiac myocytes and fibroblasts. For this purpose neonatal rat ventricular myocytes and fibroblasts were cultured on plates with deformable growth surfaces and subjected to pulsatile stretch.

Our hypothesis is that the increased mechanical stimulus is sensed by specific mechanoreceptors, transduced into the cell where it could, directly or indirectly, activate the apoptotic pathway. The results from this study, however, indicate that mechanical stretch does not directly activate apoptosis in cultured cardiac myocytes and fibroblasts. Results that were based on observations of cellular and nuclear morphology, proteolytic activity of caspase-3, and fragmentation of DNA.

In conclusion, we could not find a direct link between mechanical stretch and apoptosis in an *in vitro* model system of cultured myocytes and fibroblasts. We recognize that in the working myocardium, molecular signalling cascades are much more complex than those in monolayer cultures. Particularly the effect of chronically reduced cardiac function appears to be difficult to reproduce in a cell experiment.

Specific mechanoreceptors will sense mechanical stimuli, transduce them into the cell, where they can (in)-activate a broad range of signalling pathways. Candidate structures that should sense myocardial stretch are the integrins in the myocyte cell membrane. These membrane proteins act as receptors to which extracellular proteins bind, whereby they anchor the myocytes to the extracellular matrix. Moreover, at the cellular side, the integrins bind to the cytoskeleton of the cell and to sarcomere proteins, thereby assuring a certain cell form and a regularly packaged structure of myofibrils, respectively. In chapter three we have focused on receptor-ligand interaction of integrins and subsequent signalling pathways. To examine these interactions integrins were stimulated with a soluble ligand, being a pentapeptide containing the RGD (Arg-Gly-Asp) sequence that is normally present in several extracellular matrix proteins.

Cellular calcium homeostasis is modulated in a dose dependent way by the RGD-peptide, and can be inhibited by blockers of the Ca^{2+} release channel (RyR2) of the sarcoplasmic reticulum (SR). The increase of $[\text{Ca}^{2+}]_i$ in response to RGD stimulation is rather slow and is therefore considered to be mediated by Ca^{2+} release from SR via stimulation of RyR2 conductance. Besides modulating calcium homeostasis, addition of RGD to the cultures induced a time and dose dependent increase of $[\text{NO}]_i$. NO-donors, like SNP, also induce an increase of $[\text{Ca}^{2+}]_i$, a response that is blocked by RyR2-blockers, although incompletely. It appears that NO, either via protein kinase G (PKG) activation or by NO-induced nitrosylation of RyR2 and/or L-type Ca^{2+} channels, causes increased conductance of one or both channel types. The Ca^{2+} raising effects of RGD-peptide and NO are additive, adding evidence to independent pathways that converge at the level of RyR2.

Mechanical stress (MS) plays an important role in the adjustment of the myocardium to hemodynamic overload. In addition to triggering the force-length mechanism and homeometric autoregulation, MS activates signaling mechanisms involved in hypertrophic growth of cardiac myocytes. The view that mechanical stress itself is the primary factor for hypertrophic response is supported by *ex vivo* experiments.

A striking property of the adaptability of the myocardium to enhanced workload is its ability to change gene expression, thereby altering the concentration of proteins produced. In addition, the myocardial response may include initiation of production of proteins that are not produced normally, but had been produced during fetal life. In the study described in chapter six we tried to elucidate whether the changes in gene transcription due to mechanical stress *in vitro* can be considered to be the initiation of the hypertrophic response. For that purpose the responses of a mixed population of cardiac myocytes and fibroblasts and pure fibroblasts that had been subjected to a MS protocol for 24 h were compared to their controls that had not been stretched, but were similar otherwise. The results of this experiment demonstrate that the majority of genes is either hardly expressed or does not show any change in expression after subjecting the cells to MS. Upon applying a false discovery rate (FDR) control-level at 0.005 according to Benjamini-Hochberg, MS was associated with up-regulation of 130 genes and down-regulation of only 15 genes in the mixed cell population. When these results were compared with the results obtained from a pure fibroblast cultures, it became clear that the expression of only 15 out of 145 genes was different between the mixed cell-population and the pure fibroblast-population, and these were therefore called cell-type specific. However since the mixed cell population consists of both cardiac myocytes and fibroblasts it is unclear whether the observed changes in gene expression are the result of changes in gene expression in cardiac myocytes or whether they are caused by paracrine interactions between the two cell types.

The genes that demonstrate an up-regulation include genes encoding proteins that play a role in (I) biosynthesis of phospholipids and cholesterol, the two main components of the cell membrane, (II) synthesis of extracellular matrix components, (III) myofibrillar proteins, surprisingly only myosin heavy chain genes that were non-muscle subtypes, instead of muscle β -MHC, (IV) intracellular calcium handling and (V) several genes encoding growth factors and growth factor receptors.

Although apoptosis has been mentioned in relation to myocardial hypertrophy, exposure of cardiac cells to MS for 24 h did not induce an increase in single

standed DNA (ssDNA) fragments nor a change in expression of genes encoding pro-apoptotic proteins. Apparently, apoptosis is not part of the hypertrophic process per se, but rather belongs to the transition phase of hypertrophy to heart failure, or to heart failure alone.

In summary the changes in gene expression described in this chapter, strengthen the view that MS is one of the initiation factors of the hypertrophic response observed in overloaded myocardium.

In the study described in chapter seven we set out to investigate how the stress response of cardiac cells to MS can be compared to genotoxic stresses induced by DNA damaging agents. For this purpose we choose ionising radiation (IR), which during mediastinal radiotherapy can result in cardiac tissue remodelling and diminished heart function, and ultraviolet radiation (UV) that, in contrast to IR induces high concentrations of DNA replication- and transcription-blocking lesions.

To identify and compare differentially expressed genes (upregulated or down-regulated when compared with untreated controls) in cardiac cells in response to the three stresses MS, IR or UV, cultures enriched for ventricular cardiac myocytes or fibroblasts were exposed to one of the three stressors. Differentially expressed genes were identified using Affymetrix GeneChips. Since overall changes in the expression of functionally related genes are more informative than the expression pattern of single genes, in the present study, genes were assigned to functional groups, based on biological function or on the role of a gene product, in common intracellular pathways.

In the responses to all three stressors, differentially expressed genes were mostly up-regulated ($q < 0.005$) in cardiac myocyte-enriched fractions, while in cultures of fibroblasts the majority of changed genes were down-regulated, differences that were most pronounced following mechanical stress (MS). It is important to mention that the percentage of cardiac fibroblasts in the cardiac myocyte enriched fraction that was subjected to MS was higher than the percentage of cardiac fibroblasts in the cardiac myocyte-enriched fractions that were subjected to UV or IR. The differences in gene expression level might very well be caused by the differences in level of toxicity between the three stressors applied in this study. However one must not forget the effect of paracrine signalling in the co-cultures. Especially in the myocyte-enriched co-culture that is subjected to MS paracrine signalling might have great influence.

From this study we can conclude that MS, IR and UV mainly induce stress-specific and cell-type specific gene expression profiles in neonatal rat cardiac myocyte-

enriched cultures and cultures of neonatal rat heart fibroblasts. Functional groups that show significant percentages of differentially expressed genes suggest that certain cellular pathways are activated after one or more stresses.

Besides the effect of cardiac specific stresses (MS and integrin stimulation) on both the cardiac myocytes and the cardiac fibroblasts, we also examined and described the ability of these cells to repair DNA damage. Many exogenous and endogenous stresses affect the preservation of nuclear DNA, of which the integrity is essential for the correct performance of many cellular processes. In virtually all organisms from bacteria to higher eukaryotes, several DNA repair pathways prevent the deleterious consequences of DNA damages. The DNA repair mechanisms that are operational have been examined and described extensively, but knowledge of DNA repair in terminally differentiated cells, such as cardiac myocytes, is scarce.

Chapter four is therefore devoted to the capacity of cultured cardiac cells to repair damage to their DNA. To introduce DNA damage in cultured heart cells, a frequently used technique, being radiation with ultraviolet (UV) light, is employed. A versatile repair pathway in a wide variety of cells that is involved in the removal of a broad range of structurally unrelated DNA lesions, is nucleotide excision repair (NER). NER removes bulky lesions that distort the DNA helix, including the UV-induced cyclobutane pyrimidine dimers (CPD) and (6-4)-photoproducts (6-4 PP). Two NER subpathways have been identified, i.e. global genome repair (GGR) and transcription-coupled repair (TCR). GGR removes DNA lesions throughout the genome, whereas TCR acts specifically on DNA lesions in the transcribed strand of active genes. The efficiency of repair of DNA lesions by TCR and GGR in mammalian cells depends on a number of factors, including species, cell type and type of DNA lesion.

Efficient GGR of CPD in human cells requires a protein complex consisting of two protein subunits, DDB1 (p125) and DDB2 (p48), with DNA damage binding activity (DDB) having high affinity for damaged DNA. In wild type hamster cell lines and in several primary tissues from hamsters and mice binding activity and p48 expression are absent. Furthermore, these cells are all characterized by a deficiency in GGR of CPD.

Despite the knowledge of NER in human and rodent fibroblasts and established cell lines, little is known about NER and the expression of the p48 gene in terminally differentiated cells. Therefore in chapter 4 we addressed the question of NER efficiency and cellular differentiation by focusing on expression of NER and transcription of the p48 gene in terminally differentiated myocytes and proliferating fibroblasts isolated from the same hearts of neonatal rats.

Myocytes and fibroblasts were found to carry out efficient removal of 6-4 PP but display poor repair of CPD by GGR. Furthermore both cell types were found to carry out TCR of CPD, thus mimicking the repair phenotype of established rodent cell lines. The inefficient repair of CPD at the genome overall level occurs in the absence of massive apoptosis, but goes along with an undetectable level of transcription of the p48 gene. Taken together, the selective removal of CPD from the transcribed strand of transcriptionally active genes demonstrates that proliferation status has no impact on repair phenotype: both terminally differentiated myocytes and proliferating fibroblasts from the rat heart exhibit efficient TCR and poor GGR. Moreover the poor GGR of CPD is due to the absence of p48 expression.

The studies reported in this thesis confirm that cardiac cells, both myocytes and fibroblasts have the capacity to respond adequately to exogenous stresses. The responses that have been studied in detail demonstrate that these cells possess systems that sense the stress, transmit its message to the cell interior, and alter gene expression, leading to appropriate reactions.

The use of cardiac cells in culture to study these reactions has many advantages, such as simplicity compared to a complete heart, ease of handling, simultaneous experiments in series of cultures, as well as disadvantages, such as exclusion of innervation, blood-borne factors and 3-dimensional structure. Future studies with cardiac cells in culture are encouraged, particularly those addressing the mechanosensitive pathways, paracrine interaction between fibroblasts and myocytes, and the ways the extracellular matrix becomes modified by stresses. These studies may elucidate the mechanism behind the process of ventricular remodeling observed in patients with heart failure.

9

Samenvatting

De belangrijkste taak van het hart is om het organisme levenslang, ononderbroken, van bloed te voorzien. Tijdens het leven staat ieder organisme, en tevens zijn organen, bloot aan diverse stress factoren, zowel van endogene- als exogene origine. Om te overleven moet op de juiste manier gereageerd worden op deze stress factoren. Gelukkig bezit het hart een groot scala aan mogelijkheden om zich aan te passen aan, onder andere, veranderingen in de doorstroming van het hart, veranderende belastingscondities en blootstelling aan toxische stoffen.

In de studies beschreven in dit proefschrift is onderzoek gedaan naar de reactie van hartcellen op verschillende stressfactoren, in een poging zich aan te passen aan de nieuwe situatie. Het effect van mechanische stress is bekeken in de hoofdstukken twee, zes en zeven. Hoofdstuk twee is met name gericht op de relatie tussen mechanische stress en het ontstaan van apoptose, terwijl de hoofdstukken zes en zeven gewijd zijn aan de veranderingen in genexpressie die optreden onder invloed van mechanische stress. De invloed van DNA schade op de hartcellen is onderzocht en beschreven in de hoofdstukken vier en zeven. Als laatste is gekeken naar de invloed van stimulatie van de integrines, deze resultaten zijn weergegeven in hoofdstuk drie.

Een korte beschrijving van zowel de normale functie van het hart als de verschillende celtypen die in de hartspier aanwezig zijn wordt gegeven in hoofdstuk een. De cellen die in de in dit proefschrift beschreven studies gebruikt zijn, zijn de relatief grote myocyten en de kleinere fibroblasten. De myocyten beslaan de grootste massa van het hart en zijn verantwoordelijk voor de contractiele werking. Deze cellen verliezen kort na de geboorte van het organisme het vermogen om te delen en zijn terminaal gedifferentieerd. Ongeveer 50% van de cellen in het hart bestaat uit fibroblasten, deze cellen produceren de extracellulaire matrix die verantwoordelijk is voor het instandhouden van de structuur (inclusief de stijfheid) van het hart.

Een aantal factoren, bv een te hoge bloeddruk of het niet goed functioneren van een hartklep, kunnen een chronische overbelasting in het hart veroorzaken. Om te compenseren voor deze overbelasting gaat de hartspier groeien, dit noemen we hypertrofie. Hoewel deze hypertrofie in beginsel erg gunstig is, het verlaagt immers de stress op de wand van het hart, kan het uiteindelijk, als de overbelasting blijft voortduren en zowel de aanpassing als de functionele verandering niet langer voldoen, leiden tot hartfalen. Wanneer we op cellulair niveau kijken zien we dat de term hypertrofie van de hartspier duidt op overmatige groei van de myocyten en vermeerdering van de fibroblasten.

Hoofdstuk een beschrijft kort de specifieke veranderingen die zijn waargenomen in zowel de myocyten als de fibroblasten tijdens de ontwikkeling van hypertrofie. Er wordt steeds meer bewijs gevonden dat apoptose, ofwel geprogrammeerde celdood, van de myocyten een gevolg is van mechanische overbelasting. Een vermindering in het aantal myocyten zou kunnen bijdragen aan de verslechtering van de contractiliteit van het hart. Of dit echter de primaire oorzaak of een gevolg van het progressive disfunctioneren van het hart is, is nog niet duidelijk. Om meer duidelijkheid op deze vraag te krijgen hebben we in hoofdstuk twee gekeken naar de directe invloed van mechanische stress op het ontstaan van apoptose in de verschillende hartceltypen. Voor dit onderzoek zijn myocyten en fibroblasten uit de ventrikels van neonatale ratten gekweekt op flexibele bodems die pulserend werden opgerekt. Het idee achter dit onderzoek is dat verhoogde mechanische stress waargenomen wordt door specifieke mechanoreceptoren die het signaal doorgeven aan de cel, waar het, direct of indirect, apoptose kan induceren. De resultaten verkregen door observaties aan de morfologie van zowel de cel als de kern, proteolytische activiteit van caspase-3 en fragmentatie van het DNA, duiden er echter op dat mechanische stress niet direct leidt tot apoptose in hartcellen, noch in de myocyten noch in fibroblasten. Uit deze resultaten kunnen we concluderen dat, in het gebruikte *in vitro* systeem van gekweekte myocyten en fibroblasten, geen direct verband bestaat tussen mechanische stress en apoptose. We moeten er echter wel rekening mee houden dat de moleculaire cascades in het functionerende hart veel complexer zijn dan de bestudeerde cascades in de monolayer van gekweekte cellen. Met name het effect van de verminderde hartfunctie is extreem moeilijk na te bootsen in een celsysteem.

Mechanische stimuli worden waargenomen door specifieke receptoren, die deze vervolgens vertalen en doorgeven aan de cel. Belangrijke kandidaat-eiwitten voor deze waarneming zijn de integrines in de membranen van de cel. Deze membraan eiwitten functioneren als receptoren voor extracellulaire eiwitten waardoor de cellen binden aan de extracellulaire matrix. Bovendien verbinden de integrines intracellulair het cytoskelet en de sarcomeer eiwitten waardoor de cel zijn vorm krijgt en er een regelmatige structuur van myofibrillen ontstaat.

De interactie tussen de extracellulaire eiwitten en de integrines, de zogenaamde receptor-ligand interactie, en de (in-) activatie van de signaal transductie paden die deze interacties tot gevolg hebben, zijn onderzocht en beschreven in hoofdstuk drie. Voor deze studie hebben we gebruik gemaakt van een pentapeptide, met een RGD (Arg-Gly-Asp) sequentie die normaal aanwezig is in een aantal matrix eiwitten. Dit RGD-peptide moduleert de cellulaire calcium huishouding op een

dosis afhankelijke manier en kan geblokkeerd worden met blokkers van het Ca^{2+} release kanaal (RyR2) van het sarcoplasmatisch reticulum (SR). De verhoging van de intracellulaire calcium concentratie als gevolg van RGD stimulatie treedt vrij langzaam op en lijkt het gevolg te zijn van stimulatie van Ca^{2+} release uit het SR. Naast de dosis afhankelijke verandering in de calcium huishouding veroorzaakt de toevoeging van RGD-peptide aan de celkweken een tijds- en dosis-afhankelijke verhoging van de intracellulaire NO concentratie. NO donoren, zoals natriumnitroprusside, verhogen de intracellulaire calcium concentratie en tevens kan deze verhoging, hoewel niet geheel, geblokkeerd worden door RyR2 blokkers. Het lijkt erop dat NO, hetzij via protein kinase G (PKG) activatie, of door NO-geïnduceerde nitrosylering van RyR2 en/of L-type calcium kanalen, een verhoging van de geleiding van één of beide kanalen veroorzaakt. Het feit dat de intracellulaire calcium verhogende effecten van het RGD-peptide en NO additief blijken te zijn, vormt een extra aanwijzing dat er onafhankelijke signaaltransductie paden geactiveerd worden die samenkomen op het niveau van RyR2.

Een eigenschap van het aanpassingsvermogen van het hart aan veranderende omstandigheden, is de mogelijkheid om de expressie van genen en bijgevolg de concentratie van de bijbehorende eiwitten te veranderen. Behalve dat is het hart ook in staat weer eiwitten produceren die normaal alleen tijdens de foetale ontwikkeling geproduceerd worden.

Tegenwoordig kan de expressie van vele duizenden genen in een keer bestudeerd worden met behulp van DNA micro arrays. Een techniek die is toegepast in hoofdstuk zes en zeven om de respons van de myocyten en de fibroblasten op verschillende stress factoren te bepalen. Het onderzoek beschreven in hoofdstuk zeven is uitgevoerd om te kijken in hoeverre de stress reactie van hartcellen op mechanische stress, een hartspecifieke stress, vergeleken kan worden met het ontstaan van DNA schade, een meer algemene stress. Om DNA schade te veroorzaken is gebruik gemaakt van twee verschillende soorten straling. Als eerste zijn de celkweken blootgesteld aan ioniserende straling (IR), dat in patiënten die radiotherapie ondergaan kan leiden tot een remodeling van het hart en een verminderde hartfunctie. De tweede straling die is toegepast, is UV-straling, dat, in tegenstelling tot IR, veel DNA replicatie en transcriptie blokkades veroorzaakt.

De gebruikte celkweken in hoofdstuk zeven bestonden uit een mix van zowel terminaal gedifferentieerde myocyten als delende fibroblasten uit het hart van neonatale ratten. Om aan het eind van de kweek periode de verandering in genexpressie op de verschillende stress factoren van beide celtypen afzonderlijk te bepalen is gezocht naar een methode om de beide celtypen van elkaar te scheiden.

Om een goede scheiding te bereiken is gebruik gemaakt van centrifugaal elutriatie (hoofdstuk vijf). Door de stroomsnelheid door de elutriatiekamer te variëren kan een cel-fractie rijk aan fibroblasten ($87 \pm 5\%$ zuiver) en een myocyten verrijkte fractie ($82 \pm 6\%$ zuiver) verkregen worden. Ondanks het feit dat met deze techniek de niet-myocyten goed van de myocyten gescheiden kunnen worden, is ook gebleken dat na een langere periode van kweken (4-5 dagen) het kleine aantal niet-myocyten in de myocyten verrijkte kweek, zorgde voor een vermindering in het relatieve percentage myocyten. Om die reden is een tweede elutriatie protocol ontworpen, waarbij de myocyten van de niet-myocyten pas gescheiden zijn na een periode van 4-5 dagen co-kweken. Dit laatste elutriatie protocol, waarin de cellen pas aan het eind van het experiment van elkaar gescheiden worden, heeft als grote voordeel dat de effecten van identieke experimentele condities apart bestudeerd kunnen worden in myocyten en niet-myocyten.

De reactie op de drie verschillende stress factoren is gemeten door een co-kweek van cellen bloot te stellen aan een van de drie stress factoren en te vergelijken met een co-kweek van controle cellen. Na de cel scheiding met behulp van de elutriator zijn de differentiële tot expressie gebrachte genen (omhoog- of omlaag gereguleerd ten opzichte van de niet behandelde controle cellen) geïdentificeerd en vergeleken met behulp van Affymetrix GeneChips. Vanwege het feit dat de totale verandering in expressie van functioneel gerelateerde genen meer zegt dan het expressie patroon van individuele genen, zijn de genen op de Affymetrix GeneChips ingedeeld in functionele groepen, gebaseerd op hun biologische functie of op de rol van het genproduct in algemene intracellulaire paden.

In reactie op alle drie de stress factoren zijn vele genen in de myocyt-verrijkte fractie omhoog gereguleerd ($q < 0.005$), terwijl de meeste genen, die in de fibroblasten fractie veranderde expressie vertonen omlaag gereguleerd zijn. Dit verschil kwam het duidelijkst tot uiting na behandeling met MS. Het is hierbij wel belangrijk om te vermelden dat het percentage fibroblasten in de myocyten-verrijkte fractie van de cellen die MS hebben ondergaan groter was dan het percentage fibroblasten in de myocyten-verrijkte fractie die bestraald zijn door IR of UV. Hoewel het verschil in genexpressie niveau heel goed het gevolg kan zijn van het verschil in toxiciteit van de drie gebruikte stress factoren, moeten we niet het paracrine effect in de co-kweken vergeten. Met name het paracrine effect in de aan MS onderworpen myocyten-verrijkte fractie zou grote invloed kunnen hebben op de regulatie van genexpressie.

Uit de resultaten van deze studie kunnen we concluderen dat MS, UV en IR voornamelijk stress specifieke en cel-type specifieke genexpressie profielen

induceren in fibroblasten en myocyt-verrijkte kweken van de hartjes van neonatale ratten. De functionele groepen, die verandering in expressie in een significant percentage van de genen laten zien suggereren tevens dat er bepaalde cellulaire paden zijn die geactiveerd worden na meer dan één stress factor.

De studie beschreven in hoofdstuk zes gaat verder in op de veranderingen in gen expressie die door MS geïnduceerd worden. MS speelt een belangrijke rol in de aanpassing aan hemodynamische overbelasting. Behalve dat het het kracht-lengte mechanisme en de homeometrische autoregulatie in werking zet, activeert MS signaalmechanismes die betrokken zijn bij de hypertrofe groei van de myocyten uit het hart. De gedachte dat MS zelf de primaire factor is voor de hypertrofe reactie wordt ondersteund door *ex vivo* experimenten. De studie beschreven in hoofdstuk zes is uitgevoerd om op te helderen of de veranderingen in genexpressie, die *in vitro* optreden als gevolg van MS, beschouwd kunnen worden als de initiatie van de hypertrofe reactie.

Om de reactie van gekweekte hartcellen op MS te bestuderen is de genexpressie van cellen die 24 uur een rekprotocol hebben ondergaan vergeleken met de genexpressie van een soortgelijke kweek die niet gerekt is. Het resultaat van dit experiment laat duidelijk zien dat het merendeel van de genen nauwelijks tot expressie komt en dat MS daar geen verandering in brengt. Als we een false discovery rate (FDR) van 0.005 volgens de Benjamini-Hochberg analyse hanteren, blijkt dat MS ervoor zorgt dat de expressie van 130 genen omhoog en 15 genen omlaag gaat in een celpopulatie die zowel myocyten als fibroblasten bevat. Wanneer we deze resultaten vergelijken met de resultaten van een zuivere fibroblasten kweek, blijkt dat 15 van de 145 in expressie veranderde genen verschillen tussen de zuivere fibroblasten kweek en de gemengde celpopulatie; deze genen noemen we cel-type specifiek. De gemengde celpopulatie bestaat echter uit zowel myocyten als fibroblasten en het is dus onduidelijk of de waargenomen verandering in genexpressie wordt veroorzaakt door een reactie van de myocyten op de MS, of dat het een gevolg is van een paracrine interactie tussen de beide cel-typen, die door MS is geïnduceerd.

De genen waarvan de genexpressie omhoog gaat als gevolg van MS coderen voor eiwitten die een rol spelen in (I) de biosynthese van fosfolipiden en cholesterol, de twee belangrijkste componenten van de cel membraan, (II) de synthese van componenten van de extracellulaire matrix, en (III) intracellulaire calcium huishouding. Tevens gaat de expressie van genen coderend voor (IV) een aantal groeifactoren en receptoren voor deze groeifactoren en (V) myofibril eiwitten, omhoog.

Hoewel apoptose in relatie met hypertrofie van het hart genoemd wordt, leidde blootstelling van hartcellen aan MS gedurende 24 uur niet tot een toename in het aantal enkelstrengs DNA fragmenten. Tevens werd er geen verandering in de expressie van genen coderend voor pro-apoptotische eiwitten gevonden. Deze resultaten bevestigen de eerder in hoofdstuk twee gevonden conclusie dat er geen direct verband bestaat tussen MS en apoptose. Blijkbaar is apoptose geen onderdeel van het hypertrofe proces per se, maar speelt het een rol bij de overgang van hypertrofie naar hartfalen of in hartfalen alleen. Samenvattend ondersteunen de veranderingen in genexpressie, zoals beschreven in dit hoofdstuk, de gedachte dat MS één van de initiatiefactoren is van de hypertrofe reactie in het overbelaste hart.

Naast de invloed van hartspecifieke stressfactoren (MS en stimulatie van de integrines dmv RGD eiwitten), op zowel de myocyten als de fibroblasten hebben we ook gekeken naar het vermogen van deze cellen om schade aan het DNA te herstellen. De integriteit van het DNA is van groot belang voor het functioneren en overleven van de cel. Als gevolg van reacties in de cel of van buitenaf door chemische stoffen en straling wordt DNA echter voortdurend beschadigd. Vrijwel alle organismen, van bacteriën tot eukaryoten, bezitten een breed scala aan mogelijkheden om DNA schade te verwijderen en de oorspronkelijke sequentie te herstellen om op deze manier nadelige effecten van DNA beschadigingen te voorkomen. Hoewel de werking van DNA herstel mechanismen uitgebreid bestudeerd en beschreven is voor delende cellen, is er (nog) weinig bekend van de werking van DNA herstel in terminaal gedifferentieerde cellen, zoals bv myocyten uit het hart. Het onderzoek beschreven in hoofdstuk 4 is daarom gewijd aan de vraag of gekweekte myocyten uit het hart schade aan het DNA kunnen herstellen. Om op een gecontroleerd manier schade aan het DNA aan te brengen in de gekweekte hartcellen hebben we gebruik gemaakt van een techniek die vaak toegepast wordt, namelijk bestraling met ultraviolet licht (UV). UV licht induceert voornamelijk cyclobutaan pyrimidine dimeren (CPD) en pyrimidine (6-4)-pyrimidone fotoproducten (6-4PP) in het DNA.

Een veelzijdig DNA herstel mechanisme, betrokken bij het verwijderen van allerlei soorten DNA schade, onder andere de DNA schade die ontstaat na blootstelling aan ultraviolet licht (UV), is nucleotide excissie repair (NER). NER bestaat uit twee processen die naast elkaar plaatsvinden: transcriptie gekoppeld herstel (TCR) dat verantwoordelijk is voor de verwijdering van schade in de afgeschreven streng van transcriptioneel actieve genen, en algemeen genoom herstel (GGR), dat verantwoordelijk is voor de verwijdering van schade in het hele genoom, inclusief

DNA sequenties die niet tot expressie komen, waaronder de niet-afgeschreven streng van actieve genen.

Uit onderzoek blijkt dat de mate van het herstel van UV-geïnduceerde schade niet gelijk is in de verschillende delen van het genoom. Zo worden in gekweekte knaagdiercellen CPD's snel verwijderd van de afgeschreven streng van actieve genen. Deze schade wordt echter niet of nauwelijks verwijderd van de niet-afgeschreven streng, noch uit de inactieve sequenties van het genoom. Voor de efficiënte verwijdering van CPD's door GGR is een eiwitcomplex vereist dat een beschadigd DNA bindende capaciteit (DDB) heeft en daarnaast een grote affiniteit bezit voor dit beschadigde DNA. Dit complex is opgebouwd uit twee subeenheden, DDB1 (p125) en DDB2 (p48). Wild type hamster cellen en een aantal primaire weefsels van hamsters en muizen missen deze beschadigd DNA bindende capaciteit en de expressie van p48, daarnaast worden deze cellen gekarakteriseerd door een defect in GGR van CPD.

Hoewel er al veel bekend is over NER in cellijnen en delende fibroblasten, zowel van humane- als knaagdier-origine, weten we nog weinig over NER en de expressie van p48 in terminaal gedifferentieerde cellen. In hoofdstuk 4 hebben we ons bezig gehouden met de vraag wat de invloed is van cellulaire differentiatie op de efficiëntie van NER. Om deze vraag te beantwoorden hebben we de expressie van NER en de transcriptie van p48 bestudeerd in terminaal gedifferentieerde myocyten en delende fibroblasten geïsoleerd uit dezelfde hartjes van neonatale ratten.

Uit dit onderzoek blijkt dat zowel myocyten als fibroblasten 6-4PP efficiënt verwijderen van het DNA maar dat het herstel van CPD door GGR slecht is. Beide cel-typen verwijderen bovendien CPD van de afgeschreven streng van actieve genen met TCR. Met andere woorden, myocyten en fibroblasten uit de hartjes van neonatale ratten bootsen het repair fenotype van knaagdiercellijnen na. Dit inefficiënte verwijderen van CPD van het hele genoom gaat gepaard met een lage, niet-detecteerbare, transcriptie van p48, een gen dat sinds kort geacht wordt van essentieel belang te zijn voor het verwijderen van CPD van het hele genoom.

Samenvattend kunnen we zeggen dat de selective verwijdering van CPD van de afgeschreven streng van actieve genen laat zien dat de proliferatie status van de cel geen invloed heeft op het DNA herstel fenotype.

De in dit proefschrift beschreven studies bevestigen dat hartcellen, zowel de myocyten als de fibroblasten, in staat zijn om op de juiste manier om te gaan met exogene stress factoren. De reacties die in detail bestudeerd en beschreven zijn laten zien dat beide celtypen de stress kunnen waarnemen, doorgeven aan de kern

van de cel en vervolgens de expressie van genen kunnen aanpassen, zodat de cellen op de stress kunnen reageren.

Het gebruik van gekweekte hartcellen voor het beschreven onderzoek heeft veel voordelen: het is een makkelijk hanteerbaar systeem, een versimpeling ten opzichte van het complete hart en het maakt het mogelijk om meerdere experimenten simultaan in series van kweken uit te voeren. Er kleven echter ook nadelen aan het gebruik van celkweken. Zo is het niet mogelijk om de 3-dimensionale structuur en de innervatie van het hart na te bootsen en is het erg moeilijk om de invloed van factoren uit het bloed te bestuderen. Toch blijft het in de toekomst nuttig om gebruik te maken van gekweekte hartcellen, met name om de mechanogevoelige paden, de paracrine interacties met de fibroblasten en de modificatie van de extracellulaire matrix als gevolg van deze factoren te bestuderen. Deze studies kunnen bijdragen aan de opheldering van de mechanismes die ten grondslag liggen aan de processen van vervorming van de hartkamers van patiënten met hartfalen.

Curriculum vitae

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Karliën van der Wees werd op 22 april 1973 geboren in Amsterdam. Zij behaalde in 1991 haar Atheneum diploma aan het Reformatorisch College Blaise Pascal in Zaandam. Nog dat zelfde jaar maakte zij een aanvang met de studie biologie aan de Vrije Universiteit te Amsterdam, waarin zij het doctoraal examen aflegde in december 1996. In het kader van deze studie heeft de schrijfster van dit proefschrift, onder leiding van Dr. K.W. Li en ing. R. Van Elk, een stage gelopen bij de werkgroep Moleculaire Biologie van de vakgroep Experimentele Dierkunde aan de Vrije Universiteit van Amsterdam. Na het voltooien van een specialisatie cursus onder leiding van Prof. Dr. T.A. de Vlieger bij de werkgroepen theoretische biologie en electrophysiologie eveneens van de vakgroep Experimentele Dierkunde aan de Vrije Universiteit van Amsterdam, heeft zij een laatste stage gelopen bij de werkgroep Hypothalamus II van het Nederlands Instituut voor Hersenonderzoek onder leiding van Dr. R.M. Buijs.

Van januari 1998 tot mei 2003 was zij werkzaam als assistent in opleiding bij de afdelingen Cardiologie en Toxico-Genetica (voorheen Stralengenetica) van het Leids Universitair Medisch Centrum. Op deze afdelingen werd het in dit proefschrift beschreven onderzoek verricht onder verantwoordelijkheid van Prof. Dr. A. van der Laarse, Prof. Dr. Ir. A.A. van Zeeland en Prof. Dr. L.H.F. Mullenders, gesubsidieerd door de Nederlandse Hartstichting, projectnummer 97-176. Sinds januari 2004 is zij werkzaam op de afdeling Klinische en Humane Genetica van het Leids Universitair Medisch Centrum.

List of abbreviations

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ADP	adenosine diphosphate
ANP	atrial natriuretic peptide
AT	ataxia telangiectasia
ATP	adenosine triphosphate
BER	Base Excision Repair
BNP	Brain natriuretic peptide
BS	Bloom syndrome
Ca	Calcium
CAD	Coronary Artery Disease
Caspase	cysteinyI aspartate-specific proteinases
CPD	Cyclobutane Pyrimidine Dimer
CK	Creatine kinase
CS	Cockayne syndrome
DAR	Differentiation Associated Repair
DD	death domain
DED	death effector domain
DNA PK	DNA dependent protein kinase
DSB	double strand breaks
ECs	Endothelial Cells
ECM	Extracellular Matrix
ET-1	endothelin 1
Exo 1	exonuclease 1
FA	fanconi anemi
FADD	Fas-associated death domain
FAK	Focal Adhesion Kinase
GGR	Global Genome Repair
HR	homologous recombination
IDL	insertion/deletion loop
IE	immediate early
IR	Ionizing Radiation
LDH	lactate dehydrogenase
LOH	loss of heterozygosity
MHC	myosin heavy chain
MLC	myosin light chain

MM	mismatching probe
MMP	mitochondrial membrane permeabilisation
MMR	Mismatch Repair
NBS	Nijmegen breakage syndrome
NER	Nucleotide Excision Repair
NHEJ	non-homologous end joining
PARP	poly(ADP-ribose) polymerase
PE	phenylephrine
Pi	inorganic phosphate
PM	perfectly matching probe
PNA	peptide nucleic acid
RGD	Arg-Gly-Asp motif
ROS	Reactive Oxygen Species
RPA	replication protein A
RyR	Ryanodine Receptor
SERCA	sarco(endo)plasmic reticulum Ca ²⁺ ATPase
SHR	spontaneously hypertensive rats
SL	Sarcolemma
SR	Sarcoplasmic Reticulum
TCR	Transcription Coupled Repair
TNF	tumor necrosis factor
TRADD	TNF receptor associated death domain
TRIAL	TNF related apoptosis-inducing factor
TTD	trichothiodystrophy
UV	Ultra Violet
UV-DDB	UV induced damage DNA binding protein
VSMCs	Vascular Smooth Muscle Cells
6-4PP	pyrimidine (6-4) pyrimidone photoproduct
XP	xeroderma-pigmentosum