Structure and function of the cytoskeleton in cardiac and skeletal muscle

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2004

Department of Physiological Sciences
Lund University

Structure and function of the cytoskeleton in cardiac and skeletal muscle

Muscle contraction in transgenic desmin deficient mice

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LUND
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Akademisk avhandling

som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i GK-salen, BMC, Sölvegatan 19, Lund

Fredagen den 10 december 2004, kl 9.15

Fakultetsopponent:
Docent Göran Bergström
Inst för fysiologi och farmakologi, Göteborgs Universitet

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
Department of Physiological Sciences	Date of issue: 2004-12-10
Division of Cardiovascular Physiology	
Author(s): Johanna Balogh	Sponsoring organization: Lund University

Title and subtitle

Structure and function of the cytoskeleton in cardiac and skeletal muscle
- Muscle contraction in desmin deficient mice

Abstract

We have examined the functional and structural roles of the cytoskeletal protein desmin in cardiac and skeletal muscles using a genetically modified mouse (*Des-/-*) with the desmin gene ablated. Desmin forms filaments at the Z-disks in the striated muscle sarcomere, have connections to the sarcolemma and most likely align sarcomeres and whole cells. We have shown a decreased contractile function of heart (study I) and skeletal muscle (study II) from *Des-/-* mice, indicating an important functional role of desmin transmitting the generated muscle force between cells or aligning sarcomeres. The *Des-/-* soleus skeletal muscle has an increased fatigue resistance (study II), which we interprete to be caused by a remodulation of the myosin isoform composition towards increased amount of the slow isoform. The filament lattice, examined with X-ray diffraction, is wider in *Des-/-* soleus muscles, which indicates that desmin has a structural role in anchoring the contractile filaments actin and myosin (study III). We have examined the role of a human desmin mutation (L345P) found in cardiomyopathy. The mutated desmin gene was expressed in a mouse model to study cardiac and skeletal muscle function (IV). We observed moderate signs of striated muscle myopathy. Knowledge about the intermediate filament functions is important for future treatment of desmin related myopathies. We have used the desmin deficient mouse model to examine how ATP receptor functions can be altered during cardiomyopathy. We report that a $P2Y_{11}$-like receptor is involved in mediating the ATP induced inotropic responses of the mouse heart and that this receptor function might be down-regulated in desmin deficient cardiomyopathy (V). Modulation of this receptor function could be of possible terapeutic importance.

Key words

cytoskeleton, desmin, muscle contraction, heart, skeletal muscle, transgenic mice, cardiomyopathy, ATP-receptor

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language: English
ISSN and key title	ISBN: 91-628-6314-2
Recipient´s notes	Number of pages: 126 / Price / Security classification

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Printed in Sweden
Media-Tryck, 2004
ISBN 91-628-6314-2

This thesis is based on the following studies, referred to in the text by their Roman numeral:

I. Hearts from mice lacking desmin have a myopathy with impaired active force generation and unaltered wall compliance. Johanna Balogh, Mathias Merisckay, Zhenlin Li, Denise Paulin, Anders Arner. *Cardiovascular research*. (2002) 53:439-450

II. Lower active force generation and improved fatigue resistance in skeletal muscle from desmin deficient mice. Johanna Balogh, Zhenlin Li, Denise Paulin, Anders Arner. *Journal of Muscle Research and Cell motility*. (2003) 24:453-459.

III. Desmin filaments influence myofilament spacing and lateral compliance of slow skeletal muscle fibres. Johanna Balogh, Zhenlin Li, Denise Paulin, Anders Arner. *Biophysical Journal* (2004) in press.

IV. L345P desmin transgenic mice exhibit slight morphological and functional changes of cardiac and skeletal muscles. Anna Kostareva, Gunnar Sjöberg, Shi-Jin Zhang, Johanna Balogh, Alexandra Gudkova, Peter Thorén, Anders Arner, Håkan Westerblad, Thomas Sejersen. *Manuscript* (2004).

V. Phospholipase C and cAMP-dependent positive inotropic effects of ATP in mouse cardiac myocytes via $P2Y_{11}$-like receptors. Johanna Balogh*, Anna-Karin Wihlborg*, Henrik Isackson, Anders Arner, David Erlinge. *Manuscript* (2004).

*Equal contribution to the study

Contents

this thesis are an attempt to explore the mechanical and structural roles of the desmin intermediate filaments in striated muscles.

Desmin and links to pathological conditions

The desmin gene is among the top six up regulated genes found in familial cardiomyopathy (Wang *et al.*, 2002). Aggregates of desmin filaments have been found in several cardiomyopathies, often observed as inclusions or dense bodies in histological sections of hearts with cardiomyopathy (Bertini *et al.*, 1991;Edstrom *et al.*, 1980;Hein *et al.*, 2000). It is not the over-expression of the desmin protein *per se* which causes the cardiomyopathy, as shown with cardiac specific transgenesis in mouse models (Wang *et al.*, 2002). It is rather a dysfunction of the desmin intermediate filaments since mutations in the desmin gene or in proteins assembling desmin filaments can induce both cardiomyopathy and general muscle weakness (Ariza *et al.*, 1995;Goldfarb *et al.*, 1998;Li *et al.*, 1999;Munoz-Marmol *et al.*, 1998;Sjöberg *et al.*, 1999;Sugawara *et al.*, 2000;Vicart *et al.*, 1998). The desmin related myopathies are rather rare, about 60 case studies have been reported world wide of totally 24 different mutations in the desmin gene or in proteins involved in assembly of the desmin filaments (Goldfarb *et al.*, 2004). In one case study of a 28 years old man (Ariza *et al.*, 1995), a desmin mutation leading to dysfunctional desmin filaments was associated with early cardiac arrythmia, followed by general muscle weakness, respiratory and intestinal dysfunction. This patient had a 7 amino acids deletion within the rod domain of the desmin gene (Figure 2). Transfected epithelial cells with the mutated desmin gene (the specific 7 amino acids deleted) were unable to form intermediate filaments (Munoz-Marmol *et al.*, 1998).

Another mutation in the desmin gene involving a point mutation in the leucine sequence (L345P), i.e. a thymine base is replaced with a cytosine resulting in the leucine to proline amino acid switch in the rod domain of the desmin gene at position 345, also causes a cardiomyopathy (Horowitz & Schmalbruch,

1994;Sjoberg *et al.*, 1999b). The rod domain of the desmin gene is involved in filament formation and mutations in this region of the protein can result in disruption of filament formation. When the L345P desmin mutation was transfected into cultured epithelial cells filament formation was defective (Sjöberg *et al.*, 1999a). Further, normal desmin filaments were absent in cultured satellite cells from a patient with the P345L desmin mutation (Carlsson *et al.*, 2002). We have analyzed the cardiac performance in a mouse model with the L345P mutation in the desmin gene introduced (manuscript IV).

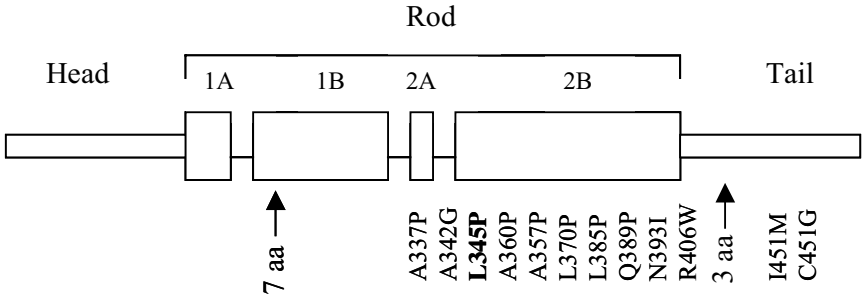


Figure 2. Schematic picture of the desmin gene with some of the reported mutations or deletions indicated.

Desmin up regulation is a feature of several myopathies of varying origin. Specific mutations in the desmin gene are rare but can cause myopathy. Not only mutations in the desmin gene but also alterations in related proteins responsible for the desmin filament formation can result in dysfunctional desmin filaments which can cause myopathy. The dysfunction is most likely related to lack of an intermediate filament structural function. This suggests that the desmin intermediate filaments are important for normal muscle function, and that changes in their structure is associated with muscle disease. The correlation between desmin filament functions and the pathophysiology of desmin-related myopathies is however not clarified.

Transgenic animal studies as evaluation of desmin filament functions

To understand the role of a specific protein, transgenic animal models are often used. A transgenic mouse with a null mutation in the desmin gene was generated, resulting in a desmin knock-out mouse, *Des-/-* (Li *et al.*, 1996;Milner *et al.*, 1996). These mice have been reported to have severe signs of cardiomyopathy with calcifications and fibrosis in the cardiac tissue (Li *et al.*, 1996;Li *et al.*, 1997;Milner *et al.*, 1996;Thornell *et al.*, 1997). Data from echocardiography measurements showed impaired systolic function in the desmin deficient mice (Milner *et al.*, 1999), suggesting that desmin intermediate filaments could be involved in force generation in the cardiac muscle. Heart rate and blood pressure measurements did not reveal any alterations in anaesthetised *Des-/-* compared to wild type (*Des+/+*) mice (Loufrani *et al.*, 2002;Milner *et al.*, 1999). The smooth muscle of the *Des-/-* mice is affected, noted as decreased generation of active force (Balogh *et al.*, 2002;Loufrani *et al.*, 2002;Sjuve *et al.*, 1998;Wede *et al.*, 2002). Information about the role of desmin filaments in skeletal muscle contraction is rather contradicting. Active force generation of intact soleus and extensor digitorium longus muscles is reported to be decreased (Li *et al.*, 1997;Sam *et al.*, 2000), but also an unaltered force generation is noted (Wieneke *et al.*, 2000), as well as an increased tetanic force in diaphragm and biceps femoris muscles in *Des-/-* mice (Boriek *et al.*, 2001). An increased expression of slow myosin isoforms is found (Agbulut *et al.*, 1996), but the mechanical consequences of this modulation in myosin isoform composition has not been examined. As discussed above, the role of desmin filaments in active force generation of cardiac and skeletal muscle of the desmin deficient mice has not been explored in detail.

There is no up regulation found of other cytoskeletal proteins, e.g. vimentin (Li *et al.*, 1997), in the Des-/- mice, but an up regulation of the extracellular matrix proteins decorin and osteopontin has recently been reported (Mavroidis & Capetanaki, 2002). These proteins are most likely involved in the process of calcification and fibrosis, since they are up regulated at the onset of calcification

and found to be co-localized at the calcium deposits in the tissue. Transforming growth factor-β_1 and angiotensin-converting enzyme are also up regulated and could further explain the extensive calcification and fibrosis seen in desmin deficient tissue (Mavroidis & Capetanaki, 2002).

The transgenic mice provide models for examining the desmin intermediate filament function in muscle. In the studies included in the present thesis we have used the desmin knock-out mice (*Des-/-*) to study the mechanical and structural functions of the desmin filaments in cardiac (study I) and skeletal (study II, III) muscles. The mice also give information on disease in man and the pathophysiology of *Des-/-* muscles would be a model for human disease with lack of, or dysfunctional, desmin intermediate filaments. We have also examined mice with over-expression of L345P mutation in desmin as a model for a specific desmin mutation in man (study IV). The alterations in receptor populations and pharmacological responses in desmin related cardiomyopathy are not characterized. As a part of an investigation of inotropic purinergic receptor responses in the mouse heart, we examined possible alterations in purinoceptor responses of the cardiomyopathic *Des-/-* heart (study V).

Cardiac contractility and purinergic receptor stimulation

ATP is released in the heart as a co-transmitter of noradrenaline from sympathetic nerves and it causes an increased contractility of cardiomyocytes (Danziger *et al.*, 1988;Mei & Liang, 2001;Podrasky *et al.*, 1997;Vassort, 2001;Zheng *et al.*, 1996). The release of ATP from cells (e.g. vascular smooth muscle cells, endothelial cells, platelets, red blood cells, cardiac cells, inflammatory cells) is increased during ischemia or mechanical stress (Clemens & Forrester, 1981;Gordon, 1986;Podrasky *et al.*, 1997;Vassort, 2001). A release of UTP from the heart during ischemia has recently been reported (Erlinge, 2004). The purinergic receptors mediating the ATP, ADP, UTP and UDP-responses are divided into two groups: the intrinsic ion channels, P2X receptors, and the G-protein-coupled P2Y receptors

Purinergic receptors – connections to pathologic conditions

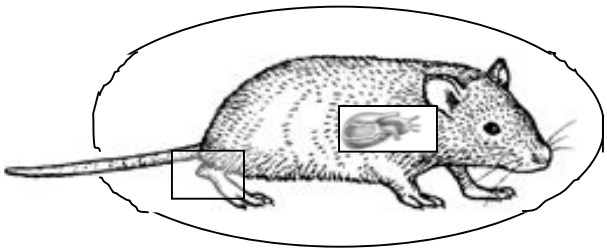
Noradrenaline and ATP are released from sympathetic nervous system when there is a demand for increased cardiac output, e.g. during exercise. In pathological hypoxic conditions, elevated levels of nucleotides are noted in the heart (Clemens & Forrester, 1981;Vial *et al.*, 1987). It has previously been reported that rats with congestive heart failure have up regulated $P2X_1$ and $P2Y_2$ receptor mRNA levels (Hou *et al.*, 1999a). However, expression of β_1-receptors is shown to be down regulated in congestive heart failure (Ungerer *et al.*, 1993). These previous findings encouraged us to study the response to stable ATP stimulation in cardiomyocytes from desmin deficient, *Des-/-*, mice with cardiomyopathy (Li *et al.*, 1996;Milner *et al.*, 1999) to explore if purinergic receptor responses are altered in desmin related cardiomyopathy (study V).

AIMS

The general aim of the studies included in this thesis was to examine the mechanical functions of the desmin intermediate filaments in striated muscle.

- Are the desmin intermediate filaments important for active force generation in cardiac and skeletal muscle?
- Is the skeletal muscle contractile phenotype altered in desmin deficient mice?
- Are the desmin filaments important for the arrangement of contractile filaments and for maintenance of cell volume?
- Does desmin contribute to mechanical connection between sarcomeres and the sarcolemma?
- What are the mechanical consequences in striated muscle of the L345P desmin mutation?
- How does ATP affect the contractility of mouse cardiomyocytes and which receptors are involved?
- Is the sensitivity towards ATP-analogues altered in desmin related cardiomyopathy?

Summary of methods



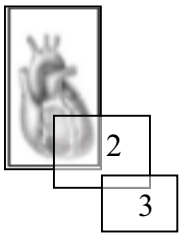
In vivo studies:

Cardiac function – Echocardiography [IV]

Skeletal muscle function and
Behavior studies - SHIRPA-protocol [IV]

In vitro studies:

Heart



1a. Cardiac function – Langendorff/isolated heart[I]
1b. Histology–Staining of paraffin embedded or frozen sections[I,IV]
1c. Protein content - SDS-gel electrophoresis [I, IV]
1d. mRNA expression - RT-PCR [IV,V]

2a. Force generation - Trabecular preparations[V]
2b. Skinned trabecular preparations[I]:
- Calcium-sensitivity
- Force generation
- Rate of force development

3. Cardiomyocyte function – contractility of isolated cells

Skeletal muscle

1a. Skeletal muscle function – Isolated intact muscle[II, IV]:
- Force generation
- Fatigue resistance

1b. Protein content - SDS-gel electrophoresis[I, II]

2. Skinned muscle preparations (bundles and single cells)[II,III]:
- Calcium-sensitivity
- Force generation
- Structural analysis- X-ray diffraction

The study where the respective method is used is indicated with Roman numerals. Some of the experiments were performed by our collaborators at Dept. of Cardiology, Lund; Karolinska Institute, Stockholm and at University of Paris. This is indicated in the text. All other experiments were performed in Lund and at HASY-lab, Hamburg by the Lund research group.

Transgenic mice

Two transgenic mice models were used: Desmin knockout mice (studies I, II, III, V) and mice with over-expression of a mutated desmin gene, L345P (study IV). These mice were obtained in collaboration with laboratories in Paris and Stockholm and examined in comparison with their wild type controls. In experiments on normal cardiac muscle (study V), NMRI mice were used. All experiments were approved by the local animal ethics committee and the investigations conform to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Transgenic desmin deficient mice (*Des-/-*) were obtained from the laboratory of Drs. Paulin, Li and coworkers at University Paris VII (Li et al 1996). A desmin gene construct was inserted in frame in a vector, which was transferred to embryonic stem cells via electroporation. Cells, with one mutated unfunctional desmin gene incorporated via homologous recombination, were microinjected into blastocysts of C57BL6 mice. The homozygous *Des-/-* mice were obtained from back crossing of the chimeric mice. The mice with two wild type alleles (*Des+/+*), i.e. with normal desmin content, served as controls. These mice were used in studies I, II, III and V.

The L345P desmin mutated transgenic mice (DM), used in study IV, were generated in the laboratory of Dr. Sejersen and coworkers at Karolinska Institute. A leucine to proline mutation was induced by a point mutation from a C (cytosine) to a T (thymine) base with site directed mutagenesis (Sjöberg *et al*., 1999). The desmin construct was tagged with a hemagglutinin sequence (HA-tag) for detection. The construct was inserted downstream of the desmin promoter. The whole complex was then purified and microinjected into pronuclei of J-129 mouse egg cells. A stable transgenic mouse line over expressing the L345P mutated desmin was obtained after mating with C57BL6 mice.

Echocardiography

Echocardiography is a non-invasive tool for imaging the heart and surrounding structures in the living anesthetized animal via an ultrasound examination. This method is recently adapted to be used on mice, which provides a further tool for the investigation on heart function of transgenic animals. The diameters in the left ventricle during systole and diastole are measured, as well as the systolic and diastolic posterior wall thickness. Left ventricular shortening fraction can also be estimated. Echocardiography was used in study IV on DM mice and these experiments were performed by the collaborators at the Karolinska Institute, Stockholm.

Behavioural and functional analysis (SHIRPA)

The SHIRPA protocol (Rafael *et al.*, 2000) includes several tests providing information about motor and neurological defects in the living animal. These tests were used for general phenotype characterization of the DM transgenic mice, and were performed by the collaborators in Stockholm. An included test is the *wire maneuvre test*, where the mouse is suspended and thereafter allowed to grab a metal wire. Other tests are *touch escape test* and *limb tone test* where the mouse is reacting on the hand approach and where the resistance of the hind limb is examined (study IV).

SHIRPA:
SmithKline Beecham Pharmaceuticals
Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit
Imperial College School of Medicine at St Mary's
Royal London Hospital, St Bartholomew's and the Royal London School of Medicine
Phenotype
Assessment

Langendorff heart preparation

This is a method to study the cardiac performance using pressure measurements of the left ventricle during retrograde perfusion of the isolated heart (Langendorff, 1895). A schematic drawing of the experimental set-up is shown in Figure 4.

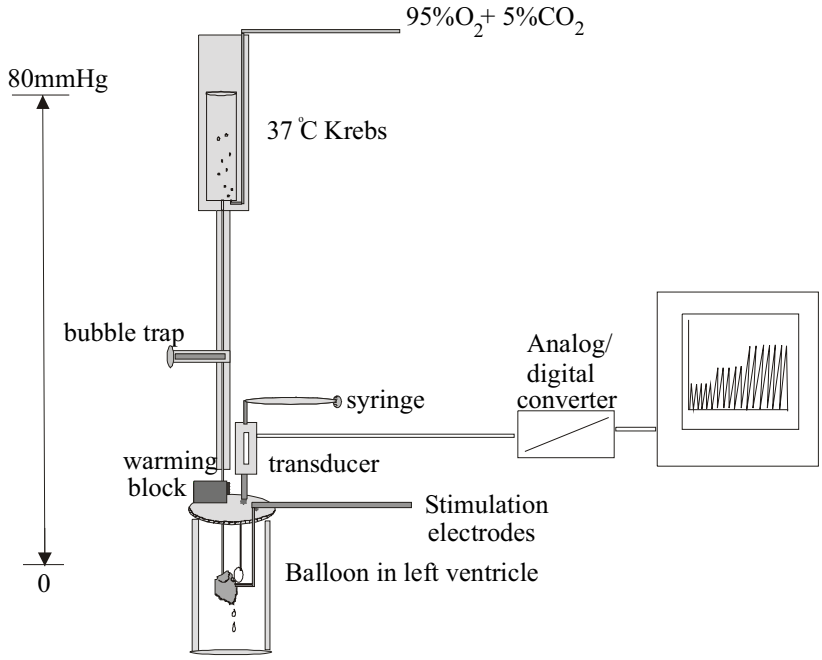


Figure 4. Schematic description of a Langendorff isolated heart preparation. The ventricular diastolic and systolic pressures are measured via a fluid filled balloon inserted in the ventricle. The filling of the balloon is controlled with a syringe and the pressure is recorded by a pressure transducer.

In these experiments, the heart is excised and perfused via aorta with Krebs-Henseleit solution to remove coagulating blood. Thereafter beating of the heart is stopped and the heart is relaxed in a high potassium containing Krebs solution (cardioplegic solution). After mounting in the experimental apparatus, the heartbeats were initiated by perfusion with 37°C oxygenated (O_2/CO_2 95%/5% resulting in a pH of 7.4) Krebs-Henseleit solution at a perfusion pressure of 80 mmHg. At this pressure the aortic valves close and perfusion is thereby achieved via the coronary arteries, which open just above the aortic valves. The pressure development is dependent on the beating frequency; therefore the beating frequency of the heart is set with electrical stimulation to enable adequate comparison of cardiac performance between different hearts. A small fluid filled balloon is inserted in the left ventricle and connected to a pressure transducer to monitor the left ventricular diastolic and systolic pressures. The difference

between the systolic and diastolic pressure is the developed pressure, also referred to as the active pressure. At the end of each experiment the heart and the left ventricle are weighed and the ventricular wall diameter is calculated to further calculate the active stress, i.e. force per cross-sectional area of the left ventricular wall (Brooks & Apstein, 1996). This method is a convenient tool when analyzing transgenic animal models regarding heart function and was used in study I and IV.

Isolated intact trabecular preparations

Intact trabecular preparations are excised from the ventricles and mounted with silk thread between a fixed rod and a force transducer to analyse the contractility after stimulation with substances of interest. The preparations were electrically stimulated and held in 37°C oxygenated Krebs solution. This technique enables examination of cardiac contractility of small intact tissue preparations in minimal solution volume (study V).

Isolation of cardiac cells

Single cardiomyocytes are isolated by perfusion of the intact heart with a collagenase containing solution. The heart is first rinsed and perfused via the aorta with 37°C oxygenated Krebs solution and thereafter perfused with a 37°C oxygenated Hepes-buffered solution with low calcium concentration and addition of collagenase. The heart is then cut into pieces and incubated at 37°C during shaking in Hepes-buffered solution with intermediate calcium concentration. The cells are washed and transferred to increasing calcium levels from 0.1 mM to 1.8 mM in several steps (Zhou *et al.*, 2000). The cells were kept in Krebs solution at 22°C and electrically stimulated in a cuvette on the stage of an inverted microscope. The contractility is evaluated by analyzing the shortening of the cells during contraction (Figure 5). This is made using an image processor and a video recording system. The video signal is digitized and an edge detection system is used to analyze the cell shortening (study V).

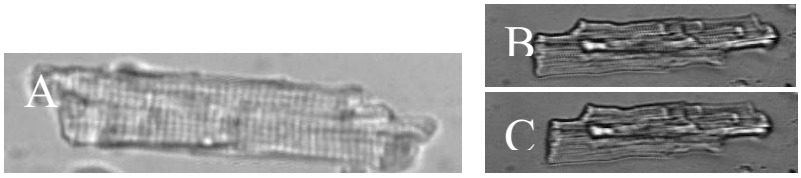


Figure 5. Panel A shows a relaxed cardiomyocyte, panel B and C shows a cardiomyocyte in a relaxed and contractile state respectively.

Fatigue stimulation on isolated skeletal muscle

Intact soleus muscles with tendons are dissected from mice. The muscle was attached vertically between a force transducer and a metal hook and stimulated electrically in an oxygenated Krebs solution at 22^{o}C. In the fatigue model used, i.e. repeated tetanic stimulation (Westerblad *et al.*, 1997), the muscle is repeatedly stimulated with 1500 ms tetani and with increasing train rate from 0.1 to 0.4 s^{-1}. Stress (active force per cross-sectional area) was calculated from the maximal force developed by the stimulated muscle, divided by the cross-sectional area of the muscle derived from the weight and length of the muscle. The decrease in force due to increased train rate of stimulation is the muscular response to fatigue. The fatigue recovery is calculated as the time the muscle needs to gain a force generation similar to that at low train rate, when train rate is altered from high to the low initial value. This protocol was used for experiments in study II. A similar protocol was used for experiments performed in Stockholm on DM mice (study IV).

Skinned muscle preparations

Chemically skinned muscle preparations are permeabilized, i.e. the plasma membrane is partly removed, with detergents such as Triton X100 or glycerol and calcium chelating, EGTA (Goldman *et al.*, 1984;Morano *et al.*, 1995). The advantage of this technique is that the intracellular calcium concentration directly can be controlled. The preparations can be stored in a glycerol solution for about 1

month at –20°C. The skinned preparations enable us to investigate the function of the contractile machinery regarding maximal active force generation, calcium sensitivity and rate of tension development in more detail. Using osmotic compression with high molecular weight dextran the compressibility of the cells can be examined. By removing ATP a rigor state can be obtained. The stable relaxed and rigor states obtained in skinned muscle are an advantage for the X-ray diffraction measurements (see below). We used skinned cardiac trabecular muscle preparations to determine active force generation, Ca^{2+} sensitivity and rate of tension development in study I. Skinned single skeletal muscle cells and fibre bundles from *m. soleus* and *m. psoas* were used in study II and III.

Rate of tension development

A substance that is chemically inactivated and instantly activated when UV-light breaks the sterical bindings, p^3-1-(2-nitrophenyl)-ethyladenosin-5′-triphosphate (caged ATP), can be used to study the rate of biological reactions. In principle, caged-ATP is introduced into a skinned muscle in rigor in the presence of Ca^{2+}. The UV-light flash releases ATP and a rapid contraction, reflecting the rate of the force-generating actin-myosin cross-bridge transitions, is developed. We have used the caged-ATP technique to estimate the rate of force generation of contracting skinned trabecular muscle preparations from *Des-/-* and *Des*+/+ in study I.

X-ray diffraction

Small angle X-ray diffraction patterns reveal information about the filament structure and distance in the muscle. The X-ray diffraction studies were performed at HASY-lab at Desy (Deutsche Elektronen-Synchrotron) in Hamburg, Germany. In the storage ring positrons are accelerated at high speed in vacuum tubes. When they are bent from their track by bending magnets, a strong synchrotron light is emitted. The X-ray light is focused on the muscle and the structures in the muscle function as a grating, scattering the light, which creates a diffraction pattern that

can be recorded (Figure 6B). The diffraction patterns are recorded with a detector or 2-dimensional image plates for further analysis. The horizontal axis of the pattern (when the muscle is mounted vertically) is denoted equatorial and the position of the distinct reflections are inversely related to the distance between filaments. As seen in Figure 6, the regular arrangement of thick and thin filaments in striated muscle gives rise to two strong equatorial reflections, 1.0 and 1.1. These have a spacing of about 42 and 23 nm. If the filaments move apart, i.e. increased filament distance, the reflections in the x-ray pattern move inwards closer to the origin. This method is used in study III.

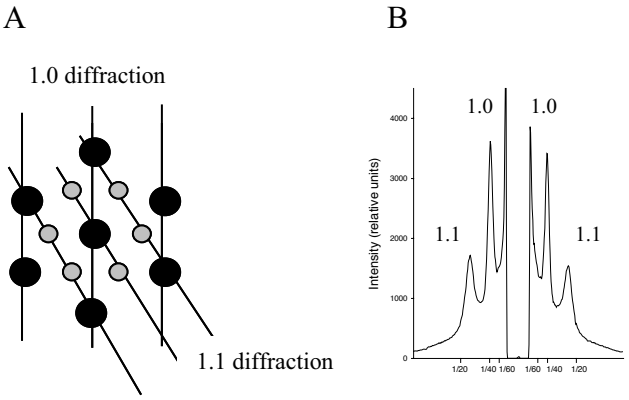


Figure 6. Panel A shows a model of the myosin (black) and actin (grey circles) arrangement in a cross section of a striated muscle. The 1.0 and 1.1 equatorial reflections indicated in the picture originate from light scattered through the spacing between myosin and actin filaments. Panel B shows an original recording of the 1.0 and 1.1 patterns.

Gel electrophoresis and Western blot analysis

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and Western blot were used to analyse the protein content in tissues. Muscle tissue is homogenized; proteins are denaturated and thereafter separated according to molecular weight on polyacrylamide gel. To study the presence of one specific protein, antibodies are used for immunological detection (Western blot). Proteins are first separated on a polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane is blocked for unspecific binding of the antibody,

incubated with a primary and secondary antibody for detection using chemiluminiscence. These techniques have been used for determination of the amount of contractile components myosin and actin and the cytoskeletal protein desmin in muscle tissue from desmin deficient, *Des-/-* and their wild type control mice *Des+/+* (studies I and II). The amount of contractile and cytoskeletal proteins after the skinning procedure was also analysed (study II, III). Western blot analysis was used in the detection of the transgenic protein (study V).

Histological staining

Tissue is fixed in paraform aldehyde or instantly frozen in Tissue Tec before sectioning into 5-8 μm thick preparations to be subsequently stained. Collagen content in hearts from *Des-/-* and *Des+/+* mice was analyzed by staining paraffin sections with 0.1% Sirius red (study I, analysis performed by collaborators in Paris). Sections from L345P desmin mutated, DM, muscles were stained with anti-desmin antibody or stained for lipids and collagen (Van Gieson, Sudan Black and Gomori trichrome staining) to visualize abnormal structures (study IV, analysis performed by collaborators in Stockholm).

cAMP measurements

Isolated cardiomyocytes were held at 37°C for 1 hour before stimulated with stable ATP analogues to examine the intracellular cAMP levels using the cAMP Enzyme Biotrak (EIA) System (Amersham Biosciences) (study V, analysis performed by collaborators at Dept of Cardiology, Lund university).

mRNA extraction, reverse-transcription (RT)- and real-time PCR

To analyze the expression of specific proteins mRNA extraction, RT-PCR and real-time-PCR techniques were used. The analyses were performed by collaborators at Dept of Cardiology, Lund. Total mRNA was prepared from isolated cardiomyocyte to analyze the expression of P2Y-receptors using RNAeasy column (Qiagen) (study V). The mRNA was then reverse-transcribed to

cDNA using Multiscribe RT Kit (Qiagen) before being amplified with real time-PCR (LightCycler) and quantified using LightCycler software (study V). Real time-PCR reveals the production of the template during each PCR cycle via incorporation of a fluorescent dye (CYBR Green) in the DNA amplification (Higuchi *et al.*, 1992;Higuchi *et al.*, 1993) as opposed to the endpoint detection in normal PCR reactions.

Results and Discussion

Desmin in different muscle types

Desmin is highly expressed in the urinary bladder but also prominent in striated muscle, where it is most abundant in the heart muscle. Using quantitative SDS-gel electrophoresis we show that the soleus muscle contains about twice the amount of desmin compared to the psoas muscle (study II: Table 1). Figure 7 illustrates a varying desmin content in different muscles from wild type mice. These results show that the expression of desmin is variable between different muscle types, which possibly could be related to different mechanical and structural functions of the intermediate filaments in smooth muscle, slow and fast skeletal muscles and in the heart.

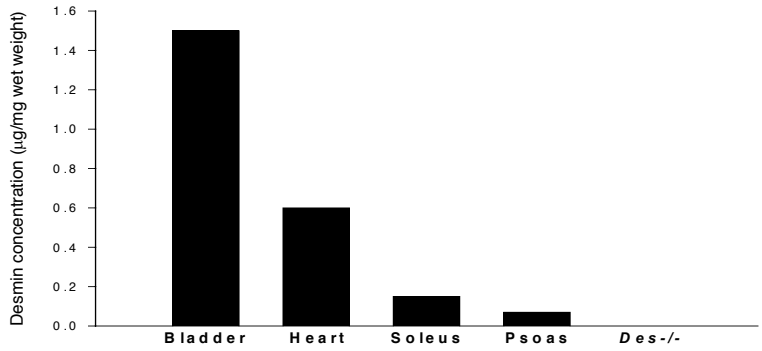


Figure 7. The native content of desmin is compared between bladder smooth muscle, heart, soleus and psoas muscles tissues from wild type and *Des-/-* mice. Desmin is absent in all tissues of the *Des-/-* mouse.

Our results from the *Des-/-* muscles along with previous findings show that desmin is absent and there is no alterations in the expression of the contractile filaments actin and myosin (study I: Table 1 and study II: Table 1) or replacement of desmin by other intermediate filaments, i.e. vimentin and synemin (Li *et al.*, 1997;Milner *et al.*, 1996) when desmin is ablated. We also showed that the expression of titin, a protein anchoring the myosin filaments (Wang *et al.*, 1979),

was not altered in the *Des-/-* skeletal muscles (study III), which is consistent with previous reports (Anderson *et al*., 2002). Previous studies have shown a shift in myosin isoform composition towards higher expression of a slow β-myosin type (Agbulut *et al.*, 1996). These results might indicate that the striated muscle of *Des-/-* mice is adapted towards a slower phenotype. This was examined in the fatigue experiments as discussed below (study II).

General phenotype characteristics of transgenic desmin knocked-out mice

The *Des-/-* mouse has a lifetime (~1 year) which is about half of their wild type controls. A major characteristical finding seems to be a cardiac myopathy, which is not associated with alterations in blood pressure or heart rate (Loufrani *et al.*, 2002;Milner *et al.*, 1999). A detailed structural examination has revealed fibrosis and calcifications of the hearts from *Des-/-* (Li *et al.*, 1996;Li *et al.*, 1997;Milner *et al.*, 1996;Thornell *et al.*, 1997) and an impaired cardiac function has also been suggested based on *in vivo* data (Milner *et al.*, 1999). The isoform composition of the contractile filament myosin is shown to be altered in the *Des-/-* cardiac and soleus muscles, with increased expression of a slow myosin isoform, type β in cardiac and type I in skeletal muscle (Agbulut *et al.*, 1996). As presented in the Introduction, the published data regarding active force generation of skeletal muscle are somewhat contradictory. Generation of active force in intact skeletal muscles is reported to be decreased (Li *et al.*, 1997), unchanged (Wieneke *et al.*, 2000) or even increased (Boriek *et al.*, 2001).

In our studies of the functions of intermediate desmin filaments in force generation of striated muscles we analyzed cardiac and skeletal muscles from *Des-/-* and compared with their wild type controls *Des+/+*. The *Des-/-* mice were somewhat smaller than their age-matched controls (Studies I-III), and we found clear evidence of calcification on the cardiac surface. The heart weights were increased in *Des-/-* mice consistent with previous studies (Sjuve *et al.*, 1998), suggesting a

cardiac hypertrophy. A major aim was to examine if these changes in cardiac structure were associated with alterations in cardiac function. *In vivo* data on skeletal muscle function are limited, although previous reports have suggested decreased performance in muscle endurance and motor coordination (Li *et al.*, 1997) and impaired performance in swimming trials (Milner *et al.*, 1999), where 50% of the *Des-/-* mice could not complete the exercise and died during swimming. *In vitro* data on muscle function in the *Des-/-* mouse are not very conclusive as discussed above, and we therefore examined the muscle function *in vitro* and found the active force generation to be impaired. This suggests that a weaker cardiac muscle and slightly weaker skeletal muscles can be characteristics in this type of desmin myopathy.

Functions of desmin intermediate filaments in cardiac contraction

The isolated heart in Langendorff preparations enables examination of cardiac muscle function, without interference from vascular system or hormonal and nervous regulatory systems. The force generated by the cardiac muscle, measured via a balloon inserted in the left ventricle, was lower in the *Des-/-* mice (Figure 8).

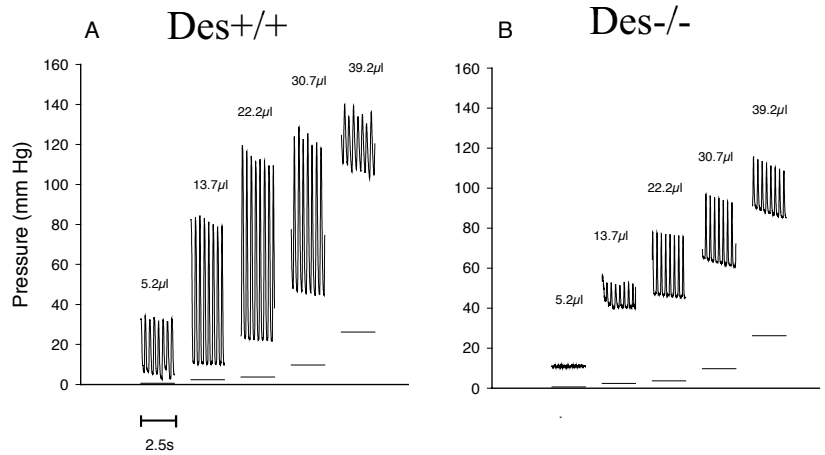


Figure 8. Pressure recordings from a balloon inserted in the left ventricle of *Des+/+* (panel A) and *Des-/-* (panel B) mouse hearts in Langendorff preparations. The volume of the balloon is increased in fixed steps as indicated in the diagram (modified from study I).

The developed pressure (i.e. systolic – diastolic pressure) and the calculated wall stress (tension in the wall divided by cross sectional area of the wall) were about 50 and 45 % lower in the *Des*-/- hearts (study I: Figures 4 and 5D-E). These results show that the cardiac muscle in the wall of the heart from mice lacking desmin has an impaired active force generation, suggesting that the desmin intermediate filament system is important for normal contractile function of the heart.

The passive, diastolic, pressure was increased indicating a smaller ventricle volume (study I: Figure 3). However, when the passive stress was calculated by considering the left ventricle diameter and wall thickness no difference was noted between the *Des*-/- and *Des*+/+ hearts (study I: Figure 5A-C). This shows that the intermediate filaments in the cardiac cytoskeleton are not important for the passive properties of the cardiac wall. The increase in left ventricle wall thickness of the of the *Des*-/- hearts indicates a cardiac hypertrophy, possibly as a compensatory mechanism developing increased ventricle muscle weight due to the decreased active force generation of *Des*-/- cardiac cells, which is induced by the lack of intermediate filaments. The calcification and fibrosis in the heart are most likely secondary effects of degenerating cardiac cells related to an impaired force generation in the heart.

The coronary flow and the spontaneous beating frequency was unaltered in the *Des*-/- hearts compared to the wild type controls (study I), which is in line with an unchanged heart rate found in anaesthetized animals (Milner *et al.*, 1999). An up regulation of the angiotensin converting enzyme, ACE, could possibly influence regulation of the blood pressure, i.e. result in an increased pressure. However, previous studies report an unchanged blood pressure in anesthetized *Des*-/- mice (Lacolley *et al.*, 2001) and the increased ACE might thus be involved in inflammatory processes, as suggested by Graninger *et al*. (Graninger *et al.*, 2004), who showed that ACE-inhibitors decreased inflammatory markers. Still, ACE could also be important for blood pressure control during activity.

Force generation of permeabilized (skinned) cardiac muscle preparations

Our result using Langendorff preparations (study I) clearly showed that the active force was lower in the cardiac muscle of desmin deficient mice. To examine the contractile function in more detail, we used permeabilized trabecular preparations. We permeabilized (skinned) the cellular membrane to be able to control the intracellular calcium concentration, to better analyse the calcium regulation and active force generation of the cardiac cells. Skinned cardiac muscle preparations from *Des-/-* mice show a decreased active force generation compared to *Des+/+* when activated at maximal Ca^{2+} concentration (Figure 9). This lower maximal stress is not due to alterations in the amounts of contractile filaments since the concentrations of actin and myosin are unaltered. The lower force in the whole heart is not due to alterations in the thin filament calcium regulator systems, since calcium sensitivity of the skinned cardiac muscle preparations was unaltered in the *Des-/-* group (study I: Figure 6B). This suggests that the active force of the *Des-/-* cardiac muscle is low although the contractile components are present and regulated in a normal way. We propose that one important mechanism for the reduction in active force is an impaired coupling between sarcomeres and cells in the absence of intermediate filaments. Thus, lack of desmin filaments, which are normally found concentrated at the intercalated disks between cardiac cells, results in an impaired force transmission between cells or that the sarcomers are not adequately coupled in the muscle cell.

The function of the heart *in vivo* and in Langendorff preparations could also be influenced by the rate of force generation, not only by the absolute active force. Since previous studies have shown that the slow β-myosin isoform is re-expressed in striated muscles of *Des-/-* mice (Agbulut *et al.*, 1996;Graninger *et al.*, 2004), we examined the rate of force generation and possible consequences of altered myosin expression. This was performed on skinned cardiac preparations using caged-compound technology (study I). The rate of force generation of skinned cardiac *Des-/-* preparations is not slower despite the suggested increase in slow myosin

isoform expression. The re-expression of the β-myosin isoform could be a compensatory mechanism involved in the cardiac hypertrophy in an insufficient amount for influencing the rate of force generation. This situation is different in slow skeletal soleus muscle where we find that the muscle is changed towards a more fatigue resistant slower phenotype (study II).

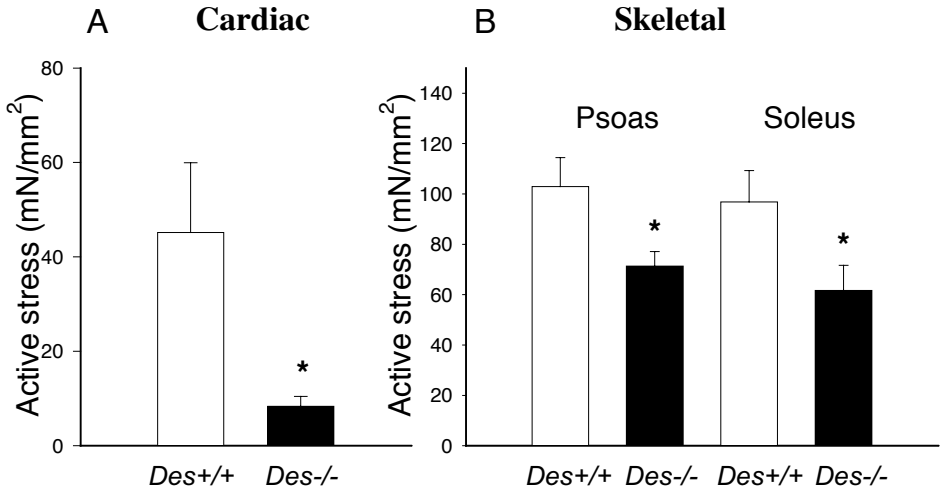


Figure 9. Active stress (force per cross sectional area) is lower in the *Des-/-* striated skinned muscles (black bars) compared to *Des+/+* (white bars). Panel A shows stress from skinned cardiac preparations and panel B stress from skinned psoas and soleus, modified from studies I and II.

Functions of desmin filaments in skeletal muscle contraction

In study II we examined the active force generation and fatigue resistance of skeletal muscles of *Des-/-* mice. The active force from the intact isolated *Des-/-* soleus muscles was slightly lower compared to *Des+/+* muscles (study II: data presented in the text). These results are consistent with previous studies by Wienecke *et al*. (2000) suggesting that intact soleus muscles from *Des-/-* mice are slightly weaker. However, the dramatic reduction of active force reported by Li et al (Li *et al.*, 1997) could not be reproduced in our study. We used a model of repeated tetanic stimulation to examine the fatigue resistance of the intact *Des-/-* soleus muscles *in vitro* (Westerblad *et al.*, 1997). After stimulation with repeated

tetani, we noted an increased fatigue resistance. The increased fatigue resistance is seen as a lower drop in force after increased train rate of stimulation and a faster recovery to the initial force level after returning to initial slow train rate stimulation (Figure 10).

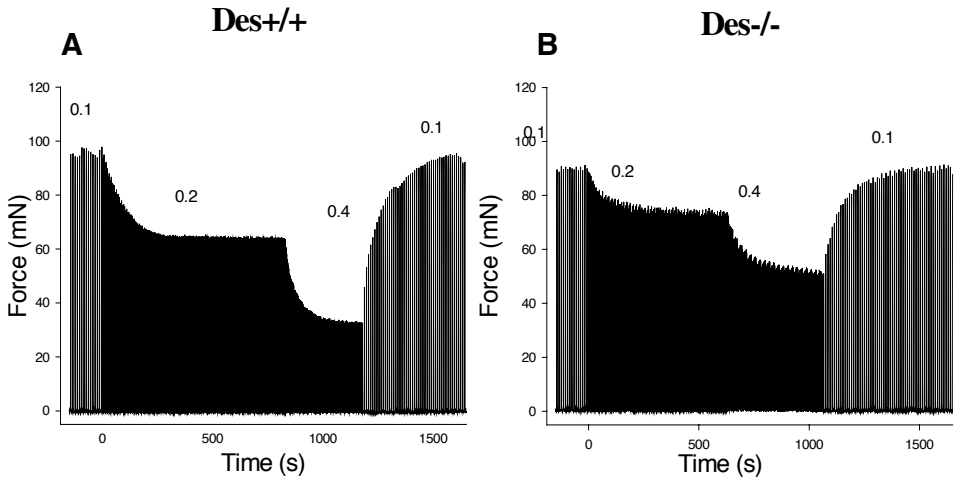


Figure 10. Force recordings from isolated soleus *Des+/+* (panel A) and *Des-/-* (panel B) muscles during fatigue stimulation at increased train rate (tetani/s indicated in the figure). Desmin deficient soleus muscles have an increased fatigue resistance, shown as less decrease in force due to increased stimulation and a faster recovery (modified from figure 1 study II).

These findings show that the increased expression of slow myosin isoforms (Agbulut *et al.*, 1996) in *Des-/-* muscle, results in a change in contractile performance of the slow skeletal muscles. Possibly this increased fatigue resistance is a compensatory phenomenon associated with the lower active force generation. In a previous study, Wienecke *et al.* (2000) showed that the *Des-/-* muscles were less resistant to high frequency fatigue. The high frequency fatigue used by Wienecke mainly challenges the Ca^{2+} -release mechanisms, and that study thus suggests that some other aspects of the striated muscle regulation are altered in the *Des-/-* muscles.

To examine possible alterations in the active force generation of *Des-/-* skeletal muscles, we analysed active stress from single skinned soleus and psoas cells

(study II: Figure 2). The muscle fibres were maximally activated with Ca^{2+}. The active stress was decreased both in the *Des-/-* soleus and psoas muscles compared to the *Des+/+* controls (Figure 9). The calcium-sensitivity was unaltered in the *Des-/-* soleus and psoas muscle cells (study II: Figure 3). These findings show that the skeletal muscles of *Des-/-* mice have a lower active force, which is present at maximal activation and not associated with altered Ca^{2+}-sensitivity at the level of the contractile proteins. We also examined the contents of contractile proteins and did not find any difference between *Des+/+* and *Des-/-* skeletal muscles (study II). These results imply that desmin filaments are important for active force generation or transmission of force in skeletal as well as in cardiac (study I) and smooth muscle (Sjuve *et al.*, 1998;Wede *et al.*, 2002) muscle. The lower force is not due to activation defects, lower amount of muscle tissue or content of contractile proteins, but rather reflects a primary defect in the mechanical coupling of the contractile system in or between the muscle cells. One model presented above for cardiac muscle is that the desmin filaments mechanically interconnect the Z-disks in the striated muscle. During active contractions sarcomeres in desmin deficient muscles are not coordinated, leading to inhomogenieties in the sarcomere length distribution, which would lower the active force. This effect would possibly be accompanied by effects of altered cell-cell or cell-tendon contacts.

Structural functions of desmin filaments in skeletal muscles

The arrangement of the intermediate filaments at Z-lines with extensions to the cell membrane suggests that desmin might be important for dimensions and compliance in the lateral direction of the muscle cell. To examine this we performed measurements of cell volume and filament distances using skinned muscle preparations (study III). Using high molecular weight dextran, which does not penetrate the filament lattice (Allen & Moss, 1987) we could compress the cells in the lateral direction. Without the connections of desmin filaments to the cell membrane, the outer diameter of the soleus muscle fibre is more compressed when exposed to high osmolarity (Figure 11, panel B). This suggests that the

coupling of desmin filaments between contractile filament lattice and the sarcolemma has a mechanical importance in regulating the cell volume. Lack of desmin intermediate filaments makes the cells easier to compress. Thus the intermediate cytoskeleton helps to retain the cell volume by introducing a cellular structure, which is able to oppose external compression of the cells. Interestingly, when the muscle is activated the cell diameter is less reduced in *Des-/-* muscle, which could reflect that the intermediate filaments are important for the transfer of forces in the lateral direction from the contractile system to the cell membranes (study III: Figure 4A-B). The alterations in skinned cell compliance upon desmin removal were only observed in the slow soleus muscles, not in the fast psoas muscle. We do not know the reason for this, possibly the difference in normal desmin content or in other mechanical properties of the muscles are involved.

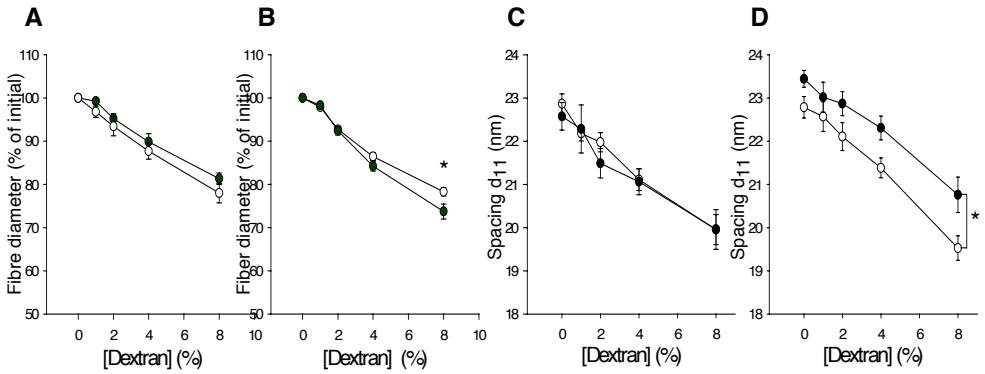


Figure 11. Relationship between single fibre diameter (panels A and B) or spacing in filament lattice (panels C and D) and osmotic compression with increasing dextran concentration of soleus (panels A, C) and psoas (panels B, D) muscles, *Des-/-* black circles and *Des*+/+ white circles. Data in panels C and D are derived from analyzing X-ray diffraction patterns (modified from figures 2 and 4, study III).

The close lateral connection between intermediate filaments and the sarcomeres suggested that the intermediate filament system could be important for the lateral arrangement of the contractile thick and thin filaments. We therefore investigated if desmin removal affected the intramuscular arrangement of the filament lattice

(study III). Using the spacing of the 1.0 and 1.1 reflections obtained from X-ray diffraction patterns we found that the filament lattice is wider in the soleus muscle of *Des-/-* mice (Figure 11, panel D). We performed additional experiments on muscles stretched to different sarcomere lengths and could determine that the "sarcomere volume" was about 12% larger in the *Des-/-* soleus muscles. This suggests that desmin filaments have a role in anchoring the contractile filaments, i.e. the myosin and actin filaments, keeping their lateral distances.

Figure 12 illustrates how we interpret the structural role of desmin in the slow striated muscle sarcomere. In soleus desmin deficient muscles, the actin and myosin filaments are wider apart because of a lack of a restraining force in the lateral direction. During osmotic diameter compression of the soleus fibre and muscle bundles, the cell diameter and filament spacing are compressed. Also in this situation the contractile filaments are wider apart in the soleus *Des-/-* muscle compared to the *Des+/+* muscle, but the cell diameter is more compressed. This suggests that the desmin filaments also are important for the mechanical connection between the contractile system and the cell membrane.

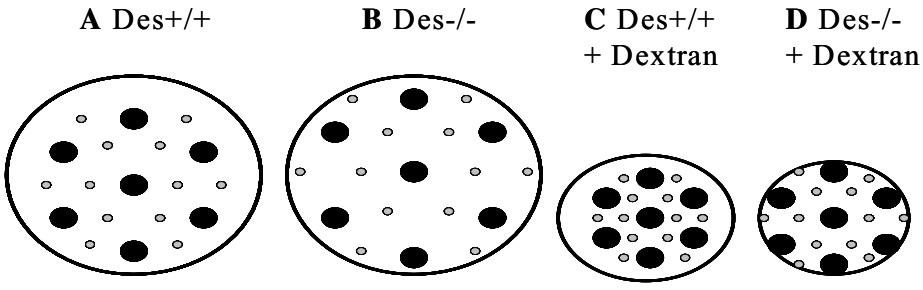


Figure 12. Panels A and B are schematic drawings of cross sectional soleus muscle fibres, showing the fibre diameter and filament arrangement of soleus *Des+/+* (panels A,C) and *Des-/-* (panel B,D) muscles. The white circles symbolize actin filaments and the black myosin, being further apart in the *Des-/-* muscle. Panels C and D show that the *Des-/-* fibre diameter is compressed more than the *Des+/+* during osmotic compression with dextran.

Coupling between force generation and desmin filaments

The psoas muscle fibres do not seem to be affected structurally by desmin removal (study III), which could be explained by the intrinsic properties of the muscle itself or by the fact that desmin content is lower in psoas compared to the soleus muscle. Interestingly, the active stress was still lower in the *Des-/-* psoas fibres compared to the psoas fibres of *Des+/+* mice (study II, Figure 9). This suggests that the wider filament spacing observed in the *Des-/-* soleus muscle, but not in the *Des-/-* psoas, is not the primary cause for the lower active force. The wider spacing is also not so large, 4%, that it would influence the active force by altering the attachment angle of the cross-bridges (Godt & Maughan, 1981;Schoenberg 1980). Further, when we compressed the spacing with dextran, we could not recover active force in the *Des-/-* soleus muscle (study III). These findings and the unaltered spacing in *Des-/-* psoas muscles indicate that the reduction in active force generation is not primarily dependent on the wider lattice arrangement. However, the wider spacing in *Des-/-* soleus muscles show that intermediate filaments have a structural role in these muscles. It has been shown that a wider filament spacing reduces muscles resistance during stretch (Edman, 1999) and the intermediate filament system might be important e.g. for muscle mechanical properties during stretching. It has been shown that *Des-/-* muscle are more resistant to stretch induced injury (Sam *et al.*, 2000), which could possibly be related to this structural/mechanical role of the intermediate filaments. Further work examining stretch-induced responses in contracting desmin deficient muscles would be interesting in this context.

All muscles, fast and slow skeletal, cardiac and smooth lacking desmin revealed a lower active force (study I, II, Li *et al.*, 1997;Loufrani *et al.*, 2002;Sjuve *et al.*, 1998;Wede *et al.*, 2002;Wieneke *et al.*, 2000). The reason for the decreased force generation in desmin deficient muscles is most likely the misalignment of sarcomeres during contraction and deficiencies in the coupling and transmission of force between sarcomeres and cells, as discussed above. The cardiac muscle seems

to be affected the most by desmin removal, since desmin deficient cardiac muscle had both a prominent decrease in force generation and pathological alterations with calcification and fibrosis (Li *et al.*, 1996;Li *et al.*, 1997;Milner *et al.*, 1996;Milner *et al.*, 1999), study I). The normal desmin expression in the heart is found at a high level in the intercalated disks (Thornell and Eriksson 1981), which suggests an important functional role of desmin filaments in the cardiomyocyte interactions. These properties and the fact that the cardiac muscle is constantly active might be important reasons for the severe cardiomyopathy in hearts from *Des-/-* mice.

Transgenic mice with the L345P desmin mutation (DM)

The transgenic mouse, DM, with the human desmin mutation L345P showed moderate signs of cardiomyopathy seen with echocardiography and histological staining of collagen (study IV: Table 2, Figure 3). This indicates that not only absence of the desmin filaments (cf. *Des-/-* mice studies I, II, III), but also a mutation in the well preserved rod domain of the desmin gene, which leads to malfunction in the desmin filament formation process, can cause desmin related cardiomyopathy. The results from SHIRPA-behaviour and functional tests show reduction in the limb muscle function, such as strength and endurance of muscle force (study IV: Figure 2).

Data from Langendorff preparations of isolated hearts from DM mice, however revealed no alterations in cardiac performance compared to control mice (Figure 13). The structural and echocardial signs of cardiomyopathy might be so moderate that it does not affect the beating heart in the Langendorff preparation.

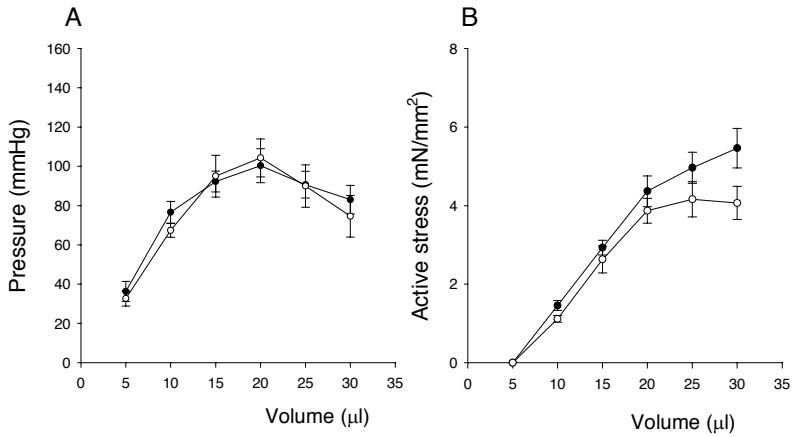


Figure 13. The isolated hearts from desmin mutated, DM, mice (black circles) showed normal heart function in the Langendorff heart preparation compared to hearts from wild type mice (white circles). Panel A shows developed (systolic-diastolic) pressure and panel B shows the active stress (tension/cross sectional area) of the left ventricular wall.

In a previous study at this laboratory using the Langendorff technique to analyse cardiac performance in mice hearts, we could detect a significant (~20%) lower developed pressure following experimentally induced cardiac infarction (data not presented), showing that the technique enables us to detect a comparable moderate decrease in cardiac performance. The unchanged cardiac performance in Langendorff preparations of DM mice suggests that the structural changes with signs of cardiomyopathy are so moderate that they do not affect the heart function when examined *in vitro*. Possibly other changes in the whole animal physiology of cardiac function *in vivo* are responsible for the altered cardiac function observed using echocardiography in the living DM animal.

Alterations in skeletal muscle function in DM mice

Signs of general myopathy were observed in the DM mice as a lower performance in the behavioural studies (SHIRPA tests) and by decreased recovery after fatigue of isolated soleus muscles (study IV, 70 weeks old mice). The soleus muscle also showed an impaired recovery after fatigue due to repeated tetanic stimulation (study IV). This is in contrast with the increased fatigue resistance observed in

desmin deficient mice and could possibly be explained by the lower degree of myopathy in DM mice. The compensatory mechanisms with increased expression of slow myosin isoforms and increased fatigue resistance might not be activated in the DM mice. In 30 weeks old DM mice we could not detect any decrease in active force generation of isolated soleus muscles. We analyzed older mice (~70 weeks) in study IV to examine a situation where the transgenic construct has been present for sufficient time. This would possibly better simulate the condition in man with this mutation, where the myopathy has a comparatively late onset (Sjöberg *et al.*, 1999). In a view of the very moderate structural and functional changes in the muscles of DM mice we have initiated a study where the DM mice are exercised (swim training) to possibly initiate activity-induced changes in the DM muscles.

Possible treatment of desmin related myopathies

It has been reported that treatment with stem cells can have a structural therapeutic effects in cardiac infarction and muscle weakness (Cossu & Bianco, 2003;LaBarge & Blau, 2002;Minasi *et al.*, 2002;Orlic *et al.*, 2001b;Orlic *et al.*, 2001a;Sussman & Anversa, 2004). An improved cardiac function after cardiac infarction following injection of bone marrow stem cells has also been reported (Check, 2004;Couzin & Vogel, 2004;Forrester *et al.*, 2003). Though, rather than becoming cardiomyocytes, it has been reported that stem cells fuse with existing cardiomyocytes (Balsam *et al.*, 2004;Murry *et al.*, 2004;Nygren *et al.*, 2004). The suggested fusion could possibly support angiogenesis, explaining some of the functional improvements reported (Sorrentino, 2004). At this stage, both the delivery of stem cells and the survival of transfected stem cells must be improved for higher success in transfection, since this also would increase the chances for improved muscle function. Recently, stem cells from the mouse embryonal aorta has been shown to move from the circulation after arterial injection into skeletal muscle (Minasi *et al.*, 2002). Following transplantation with these cells into α-

sarcoglycan deficient mice, expression of α-sarcoglycan has been detected in many of the muscle fibres from the mouse leg (Sampaolesi *et al.*, 2003), suggesting these cells as candidates for therapy of patients with muscular dystrophy. However these cells, also called mesangioblasts, have not been isolated in humans. Treatment with adenovirus or naked plasmid DNA for transfection of a stable gene construct is limited due to penetration of the virus in the muscle tissue and possible toxic effects of the adenovirus (Dickson, 1996;Hagström *et al.*, 2004;Karpati & Acsadi, 1993;Kumar-Singh & Chamberlain, 1996;Yang *et al.*, 1998;Zhang *et al.*, 2004). Although recently, delivery of gene constructs via adenoviral injection is achieved in a mouse model for Duchenne muscular dystrophy, where the dystrophin gene is ablated, with about 25% increase in the dystrophin expression in the injected diaphragm (Matecki *et al.*, 2004). In a previous study (Löfgren *et al*., 2002) we have used adenoviral transfection for insertion of genetic material into muscle preparations during tissue culture. In that study we achieved expression of a functional cre-construct in about 20% of smooth muscle cells in isolated preparations of the small intestinal outer muscle layer (the study is not included in the scope of this thesis, data not shown). The *Des-/-* mice have a genetic form of cardiomyopathy and would provide an interesting model for such treatment experiments of cardiac and skeletal muscles. For experiments on *Des-/-* mice, and future possible treatment of human patients with striated muscle myopathy the use of gene transfection seems potential, where specialized stem cells are introduced into the failing muscle tissue. Our experiments on ATP receptors shown below, suggests alterations in receptor function of the *Des-/-* heart muscle. Thus, the desmin deficient cardiomyopahtic mice would be an interesting model also for exploring pharmacological properties.

Inotropic effects of ATP in isolated mouse cardiomyocytes

In study V we have set-up a technique for analysis of contractile mouse cardiomyocytes to examine the inotropic effects of stable ATP analogues. We have had the possibility to test a stable agonist of the human $P2Y_{11}$ receptor, AR-

C67085, to specifically examine the role of this receptor in the ATP induced response. Isolation of cardiomyocytes from mice is a well defined model to study contractile function and pharmacology of cardiac muscle. One advantage in working with mice is that there are several transgenic mice strains available, and we used the desmin deficient mice characterized in studies I-III, as a model for cardiomyopathy. In study V we found an inotropic effect, i.e. an increase in the shortening of cardiomyocytes during contraction, after stimulating with AR-C67085. The inotropic effect involved activation of both cAMP and IP_3 signalling pathways. We found the rank order of inotropic stimulation to be: AR-C67085 > ATPγS > 2-MeSATP >>> 2-MeSADP = 0, presented in Figure 14A. This fits the agonist profile of the $P2Y_{11}$ receptor in human. Further, $P2Y_{11}$ is the only nucleotide coupled to both the cAMP and IP_3 signalling pathways (Communi *et al.*, 1997;Communi *et al.*, 1999). An inotropic effect was also noted when stimulating isolated intact trabecular preparations with AR-C67085, showing that the inotropic effect is also observed in isometrically contracting intact cardiac muscle. Stimulation of cardiomyocytes with the stable ADP analogue, 2-MeSADP, did not result in an inotropic response excluding the receptors $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ mediating the inotropic response. There was no inotropic response noted after stimulation with adenosine, excluding activity of adenosine receptors during ATP stimulation. In previous studies it has been stated that stimulation with α,β-MeATP does not cause any inotropic effects, excluding $P2X_1$ and $P2X_3$ as inotropic ATP mediators (Podrasky *et al.*, 1997). When we inhibited the adenylyl cyclase with SQ22563 the inotropic effect of both AR-C67085 and ATPγS was abolished in the mouse cardiocytes. If the inotropic response was mediated mainly via P2X receptors, a positive effect would be noted due to ATPγS even after blocking with SQ22563. Our results thus show that the inotropic responses following ATP stimulation in the mouse heart mainly are mediated by the $P2Y_{11}$–like receptor in the cardiomyocytes.

$P2Y_{11}$ receptor expression is yet not established in mouse, but we have found similarities with the $P2Y_{11}$ human receptor sequence in the DNA NCBI data bank, which might suggest that the receptor is expressed. We still do not have information about a cloned $P2Y_{11}$ receptor gene in mouse cardiac tissue. However our pharmacological data on the mouse cardiomyocytes show that the $P2Y_{11}$ receptor, or a $P2Y_{11}$–like receptor, is an important receptor mediating the inotropic responses due to ATP in the mouse.

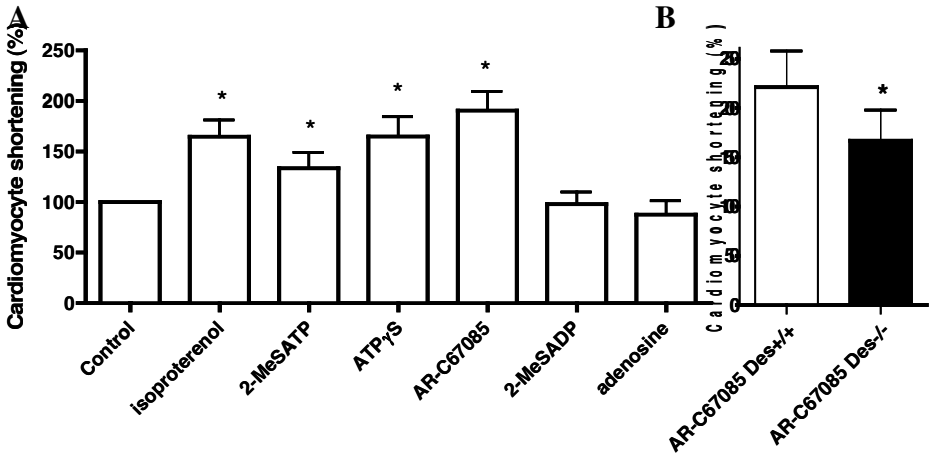


Figure 14. Isolated cardiomyocytes stimulated with stable ATP and ADP analogues. Panel A shows that the stable $P2Y_{11}$ receptor agonist AR-C67085 initiated the highest inotropic effect. Panel B shows that desmin deficient cardiomyocytes (*Des-/-*, black bar) have a decreased sensitivity to AR-C67058 (modified from study V, figures 1 and 6).

Alterations of the functions of ATP receptors in cardiomyopathy

In cardiomyopathy the $P2X_1$ and $P2Y_2$ receptors are reported to be up regulated (Hou *et al.*, 1999a). Elevated levels of nucleotides are found in the heart during ischemic conditions (Clemens & Forrester, 1981;Erlinge, 2004;Vial *et al.*, 1987). We have recently (Wihlborg and Balogh manuscript 2004, not included in this thesis) measured an increased release of UTP nucleotides during cardiac ischemia in man. The UTP release could lead to down regulation of purinergic receptors. This would be in line with a down regulation of β_1-receptors reported in heart failure (Ungerer *et al.*, 1993). Since we found that $P2Y_{11}$-like receptors are

important inotropic receptors in the mouse heart, we examined using the stable $P2Y_{11}$ receptor agonists isolated cardiomyocytes from the *Des-/-* mice to investigate if the functions of this receptor is altered during cardiomyopathy. The response to AR-C67085 was significantly decreased in *Des-/-* cardiomyocytes (Figure 14B), indicating a modulation in the $P2Y_{11}$-like receptor function, possibly via a down regulation of the expression. Cloning of the mouse $P2Y_{11}$ receptor sequence would enable analysis of receptor mRNA levels. We hope to answer the question about receptor expression in the future, when cloning of the $P2Y_{11}$ receptor in mouse is accomplished.

Possible role of ATP receptors as therapeutic targets

Since the stimulation of $P2Y_{11}$–like receptor mediates such a strong inotropic effect in the cardiomyocytes and its function in cardiomyopathy seems to be altered, it is suggested that this receptor would be a potential therapeutic target. In patients with circulatory shock it could be beneficial to improve cardiac output with $P2Y_{11}$ receptor agonists. Treatment with β-receptor blockers has been successful in congestive heart failure in lowering the cardiomyocyte metabolism and thereby unloading the heart. This could suggest that $P2Y_{11}$ receptor antagonists could be of equally therapeutic importance.

Summary

In summary, this thesis has explored the function of a key cytoskeletal protein, desmin, which forms the intermediate filaments in muscle. Ablation of the desmin gene in a mouse model (*Des-/-* mice) show that lack of this structure results in a severe cardiomyopathy and lowered active contractile force of cardiac, skeletal and smooth muscle. Our studies on cardiac muscle (study I) showed that the contractile function of the whole heart is significantly impaired and that passive (diastolic) properties are not influenced. In our study on skeletal muscle (II) we found that active force was lower and that soleus skeletal muscle adapted towards a slower, more fatigue resistant phenotype. The lower force was not associated with changes in contractile protein content and we propose a model where lack of desmin intermediate filaments is related to alterations in the sarcomere length distribution and force transmission during active contraction. Structural examination with X-ray diffraction (study III) showed that intermediate filaments are important for maintaining lateral connections between sarcomers and the cell membrane, although this structural role is not primarily important for the active force generation. Mutations of desmin can be associated with myopathy, and in study IV we examined skeletal muscles and cardiac function of a desmin mutation (L345P) introduced in mice. This mutation has been described to cause a late-onset myopathy in man. We show that minor alterations in muscle and cardiac structure/function are observed in the mice. This suggests that the desmin mutation can have a dominant negative effect. However, cardiac function *in vitro* was not influenced, suggesting that alterations *in vivo* are not due to a primary defect in cardiac muscle function. Future work on exercised animals might reveal if the mutation has a stronger effect when muscles are challenged *in vivo*. In study V we show, using a novel specific agonist, that a $P2Y_{11}$-like receptor is important mediating the ATP-induced inotropic effects in the heart and that the function of this receptor is down regulated in desmin deficient cardiomyopathy. The studies in desmin deficient mice have enabled us to address basic questions regarding the mechanical and structural roles of the desmin intermediate filaments. Since desmin

associated myopathies are important in man, results regarding e.g. mechanisms for lower force generation and stretch sensitivity, might also be important for understanding human pathology and therapy of some myopathies. Gene therapy with insertion of a functional desmin gene in muscle, e.g. with the help of adenoviral transfection, plasmid DNA delivery or injections of stem cells, could be a possible method to obtain expression of functional desmin filaments in some desmin related myopathies. The desmin deficient mouse provides a potentially interesting model for genetic cardiomyopathy and our results on the $P2Y_{11}$-like receptor suggest that an altered receptor function in the heart can be involved in this cardiomyopathy and that this receptor might be a target for pharmacological therapy.

CONCLUSIONS

- Desmin deficient mice have a cardiomyopathy with impaired systolic function and unaltered diastolic properties, and a skeletal muscle myopathy with lower active force generation.
- Desmin is important for force development in the cardiac and skeletal muscle contraction, most likely via aligning the sarcomeres and via transmission of the generated force.
- The desmin deficient, *Des-/-*, soleus muscle adapts towards a more fatigue resistant type, which correlates with an increased expression of slow myosin isoforms.
- The contractile filament lattice is wider without desmin. This structural change is most likely not responsible for the altered force generation, but might influence other mechanical properties of the muscle.
- Structural changes upon desmin removal were present in slow soleus muscles but not in psoas. This can reflect a lower desmin content in the psoas compared to the soleus muscle.
- The human L345P desmin mutation, which correlates with cardiomyopathy in humans, *per se* induce, when expressed in mice a moderate cardiomyopathy and general myopathy.
- Stable ATP-analogues generate inotropic effects in the isolated cardiomyocytes. A purinergic $P2Y_{11}$-like receptor mediates the largest inotropic response.
- Cardiomyocytes from desmin deficient mice with congenital cardiomyopathy have a decreased sensitivity to a stable $P2Y_{11}$ agonist, which could indicate a down regulation of the $P2Y_{11}$-receptors during cardiomyopathy.

Sammanfattning på svenska

I denna avhandling undersöks vilka funktioner cytoskelettproteinet desmin har strukturellt och funktionellt i hjärta och skelettmuskel. Genom att studera funktionen i hjärt- och skelettmuskler i en transgen musmodell, *Des-/-* som helt saknar proteinet desmin (delarbete I-III), kan vi uppskatta hur viktigt desmin är för muskelkontraktionen. Vi har även studerat hur en mutation i desmingenen, som tidigare har identifierats hos patienter med hjärtsvikt, påverkar den kontraktila funktionen i hjärta och skelettmuskel när den överuttrycks i en transgen mus (delarbete IV). Vi har dessutom undersökt effekten av stabila ATP-analoger på slående hjärtceller från möss (delarbete V) och undersökt om receptorfunktionen är påverkad vid hjärtsvikt genom att använda den transgena musmodellen som utvärderats i delarbeten I-III.

Desmin är bland de första proteiner som uttrycks när muskler bildas och detta protein formar filament som ingår i cellskelettet. Dessa filament finns vid Z-diskarna i sarkomeren (i änden av muskelns mindre enhet), vilken består av filamenten aktin och myosin, och som ansvarar för muskelsammandragningen. Desminfilamenten sammanlänkar flera sarkomerer i samma muskelcell samt närliggande celler och tros också ha kopplingar till cellmembranet, mitokondrier (cellens energigeneratorer) och till cellkärnan. Vid hjärtsvikt är ofta mängden desmin ökad, vilket i en del fall beror på mutationer i desmingenen eller mutationer i protein som hjälper till att bilda desminfilamenten. Ett tjugotal mutationer i desmingenen har påvisats hos människa och dessa ger upphov till hjärtsvikt och generell muskelförsvagning. Vårt syfte var att undersöka hur desminfilamenten påverkar kontraktionen i hjärt- och skelettmuskel. Vi har isolerat hela hjärtan från *Des-/-* möss och mätt slagkraften *in vitro* med en s k Langendorff metod, där hjärtat syresätts via aorta och trycket mäts via en ballong i vänster kammare. Det uppmätta pulstrycket var ca 50 % lägre och påvisar en hjärtsvikt i frånvaro av desmin (delarbete I). Även skelettmusklerna blir svagare

utan desmin, vilket vi har sett genom att stimulera isolerade preparationer av intakt soleus (vadmuskel) samt skinnade preparat, d v s kemiskt borttaget cellmembran, från hjärta, soleus och psoas (ryggmuskel) (delarbete I, II). Den lägre kraftgenereringen beror troligtvis på desmin-filamentens funktion att förankra sarkomerer och vara en länk mellan celler. Tidigare har en modulering av muskelsammansättningen rapporterats från *Des-/-* soleus muskler, vilket innebär att muskeln blir ännu mer långsam i sin fibertypsammansättning. Vi har kunnat konfirmera att den ökade mängden långsamma muskelfibrer ger en ökad resistans mot uttröttbarhet i soleusmuskel från *Des-/-* möss genom upprepad stimulering av isolerade soleusmuskler med minskade intervall mellan stimuleringarna (delarbete II).

Eftersom desminfilamenten förankrar strukturer i cellen undersökte vi vad som händer med cellstrukturen i frånvaron av dessa strukturer. Detta har vi kunnat studera med hjälp av röntgenstrålning genom att mäta hur aktin- och myosinfilamenten är arrangerade i cellerna (delarbete III). Ljusspridningen genom skelettmuskeln beror på hur filamenten är arrangerade och muskeln fungerar som ett gitter med ljusöppningar mellan filamenten. Vi observerade att dessa filament var fördelade med längre inbördes avstånd i sarkomererna när desmin är frånvarande, vilket visar att desmin håller samman filamentstrukturen i muskelcellen. Utan desmin trycks cellen lättare ihop om den utsätts för högt osmotisk tryck utifrån, vilket vi visade genom att tillsätta dextran i mediet till skinnade muskelfibrer. Detta tyder på att desminfilamenten är kopplade till cellmembranet och har strukturella funktioner som innebär att bibehålla cellens inre struktur. Denna funktion är dock inte avgörande för den lägre kraftgenereringen, eftersom muskeltyper med lägre mängd desmin, t ex psoas, också uppvisar lägre aktiv kraft, men opåverkad filamentorganisation.

Avsaknad av desmingenen har inte påvisats i humana patienter, men däremot ett antal mutationer i genen som leder till att desminfilamenten inte kan bildas

normalt. Vi har även studerat hjärtfunktionen i en transgen musmodell för en funnen mutation hos människa, där en aminosyra i desminsekvensen är utbytt (L345P). Även denna musmodell uppvisar tecken på hjärtsvikt och allmän muskelsvaghet (delarbete IV). Information om desminfilamentens detaljerade funktion är till hjälp för framtida behandling av desminrelaterade myopatier, då målet är att uppnå normalt uttryck och funktion av desmin i muskler hos dessa patienter.

När hjärtat utsätts för syrebrist, t ex vid en infarkt, utsöndras bland annat nukleotider (ATP, UTP) som ger en ökad kraft på hjärtats kontraktion. Vi har studerat isolerade hjärtceller för att undersöka hur stabila ATP-analoger påverkar hjärtkontraktionen, vilka receptorer som medierar effekten samt vilken signalväg som används i cellen (delarbete V). Vi har med hjälp av stabila ATP-analoger kunnat klargöra vilka receptorer som är inblandade vid ATP-stimulering och har även undersökt hur funktionen av dessa receptorer kan vara påverkade vid hjärtsvikt. Vi stimulerade hjärtceller från de transgena *Des-/-* mössen, som en modell för hjärtsvikt, och fann en minskad receptorkänslighet. Detta kan bero på en nedreglering av antalet receptorer och indikerar att en modulering av dessa receptorer kan vara ett terapeutiskt verktyg vid lindring av hjärtsvikt.

Zusammenfassung auf deutsch

Wir haben die funktionelle und strukturelle Rolle der Zytoskelett-Proteine Desmin im Herzen und Skelettmuskel untersucht. Diese Untersuchungen wurden in einem genetisch modifizierten Mausmodell (*Des-/-*, ohne Desmin Expression) durchgeführt. Desmin bildet Filamente an den Z-Scheiben der Sarkomere in quergestreiften Muskeln. Die Filamente verbinden Sarkomere und ganze Zellen und interagieren auch mit der Zellmembran. Wir haben gezeigt, daβ eine verringerte kontraktile Funktion im Herzen (Studie I) und Skelettmuskeln (Studie II) von den *Des-/-* Mäusen existiert. Das deutet auf eine wichtige Rolle der Desminfilamente in der Übertragung der erzeugten Muskelkraft zwischen Zellen hin. Der *Des-/-* Soleus Skelettmuskel hat einen erhöten Wiederstand gegen Ermüdung (Studie II), was vermutlich auf das erhöhte Expressionsniveau der langsamen Myosinisoform zurückzuführen ist. Röntgenstrahlen-Analysen des *Des-/-* Soleusmuskels haben gezeigt, daβ die Filamente innerhalb des Netzwerkes in einem größeren Abstand angeordnet sind. Diese Beobachtung zeigt eine strukturelle Rolle der Desminfilamente für die Assoziation zu den kontraktilen Filamenten (Aktin und Myosin) auf (Studie III). Wir haben eine spezifische Mutation des Desmingens (L345P) untersucht, die mit menschlicher Kardiomyopathie assoziert wird. Diese Mutation wird in einem Mausmodell exprimiert, um die Herz- und Skeletmuskeln zu studieren (Studie IV). Symptome quergestreifter Muskelmyopatie wurden beobachtet. Kenntnis über die Funktion der Desminfilamente ist wichtig für zukünftige Behandlung der mit Desmin in Verbindung stehehenden Myopathie. Zum Beispiel könnte Gentherapie mit Stammzellen und Expression funktioneller Desminfilamenten eine Behandlungsbasis bieten. Wir haben das Mausmodell, *Des-/-* ohne Desminfilamente, für funktionelle Untersuchung der ATP-Rezeptoren angewendet (Studie V). Die Ergebnisse dieser Studie zeigen, daβ die ATP-stimulierte erhöhte Kontraktion im Herzmuskel größtenteils durch den $P2Y_{11}$-ähnlichen Rezeptor

vermittelt wird. In Kardiomyopathie-Formen mit nicht funktionellen Desminfilamenten, ist die Funktion des ATP-Rezeptors beeinträchtigt. Modulierung der Funktion dieses Rezeptors könnte eine mögliche Grundlage für zukünftige Therapien gegen Kardiomyopathie bieten.

Acknowledgements

I would like to thank everyone who helped me and filled my days with laughter during these years as a PhD-student:

Anders Arner, my supervisor and splendid guide through science, for your neverending support and for always having time for a talk, scientific as well as fun comments on daily life.

Johan Bonnevier and Mari Ekman, for your fantastic good mood contributing to the friendly atmosphere, for endless efforts to solve every problem and of course for all interesting conversations. Without you, the lab would have been much smaller in every sense.

David Erlinge and his research group, with speial thanks to Anna-Karin Wihlborg, for interesting fruitful collaborations and good friendship.

Paul Edman, for interesting conversations about almost everything, from muscle sarcomeres to how to achieve the largest vegetable crop.

Denise Paulin, Zhenlin Li and collegues for collaboration and providing transgenic mice. Collegues in Stockholm, i.e. the research group of Thomas Sejersen.

Former collegues and present friends, Mia Löfgren, Katarina Fagher, Christina Persson, Åsa Jawarowski, Ulf Malmqvist and Rolf Sjuve for your enthusiasm and help whenever needed.

Sergio Funari, for all technical support and laughters at the beamline in Hamburg.

Present and previous students, André Erdling, Henrik Isackson, Rickard Malm, Karolina Stakeberg & Ulf Ouchterlony for contributing to the nice lab atmosphere.

Kristina Borglid, for your professional help with animal handling when I almost yielded to the mice.

All my collegues at the Dep. of Physiology, for contributing to such a nice atmosphere and making the coffee breakes longer and longer. Special thanks to Anna Wendt for being a friend and worthy opponent at the badminton court and the squash-team for keeping me in shape and sometimes letting me win.

All my friends, for all the fun and your caring support. Many friends generates many thanks, but special thanks to Clara & Bato for your grandiose hospitality and Emma & Stellan for beeing the best neighbours possible.

My parents, István & Anita for your endless care and support.
My brother Joakim & sister-in-law Fatima, for all your help and fun games of squash.

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