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Dynamic Patterns of Ventricular Remodeling and Apoptosis in Hearts Unloaded by Heterotopic Transplantation

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Abstract

Aims—Mechanical unloading of failing hearts can trigger functional recovery, but results in progressive atrophy and possibly detrimental adaptation. In an unbiased approach, we examined dynamic effects of unloading duration on molecular markers indicative of myocardial damage, hypothesizing that potential recovery may be improved by optimized unloading time.

Methods—Heterotopically transplanted normal rat hearts were harvested at 3, 8, 15, 30 and 60 days. 47 genes were analyzed using TaqMan-based microarray, western blot and immunohistochemistry.

Results—In parallel with marked atrophy (22% to 64% volume loss at 3 resp. 60 days), expression of myosin-heavy-chain isoforms (MHC- α / β) was characteristically switched in a time-dependent manner. Genes involved in tissue remodeling (FGF-2, CTGF, TGF β , IGF-1) were increasingly upregulated with duration of unloading. A distinct pattern was observed for genes involved in generation of contractile force; an indiscriminate early downregulation was followed by a new steady state below normal. For pro-apoptotic transcripts bax, bnip-3, cCasp-6 and -9, mRNA levels demonstrated a slight increase up to 30 days unloading with pronouncement at 60 days. Findings regarding cell death were confirmed on the protein level. Proteasome activity indicated early increase of protein degradation, but decreased below baseline in 60 days-unloaded hearts.

Conclusions—We identified incrementally increased apoptosis after myocardial unloading of the normal rat heart, which is exacerbated at late time-points (60 days) and inversely related to loss of myocardial mass. Our findings suggest an irreversible detrimental impact of long-term

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Disclosures

The authors have no disclosures.

unloading on myocardium that may be precluded by partial reloading and amenable to molecular therapeutic intervention.

1. Introduction

Mechanical unloading of the heart with a ventricular assist device (VAD) occasionally leads to functional recovery of the native heart¹, a phenomenon that has spurred a multitude of studies into underlying mechanisms and possible enhancement of this recovery. These have identified multiple beneficial effects summarized as “reverse remodeling”²: It consists of regression of pathological cardiomyocyte hypertrophy and improvement of several key properties of cardiac myocytes, such as calcium cycling, excitation-contraction coupling and response to adrenergic stimulation². But, it is appreciated that the loss of hemodynamic load is not only beneficial for the failing heart, but adaptive changes include myocardial atrophy and fibrosis that can negatively impact the potential for functional recovery³. The regulatory networks responsible for functional and structural changes in the unloaded heart are similar those active in pathological hypertrophy that leads to heart failure⁴.

We, amongst others, have shown in an experimental setting that unloaded hearts undergo rapid loss of myocardial mass, generating detrimental effects such as diminished cardiac output and increased myocardial stiffness, while contractile function seemed to be preserved⁵. These changes are time-dependent and studies revealing the precise dynamics of unloading-induced remodeling are still required. For instance, the role of apoptosis and/or necrosis has been addressed several times, but with inconsistent results^{6, 7}. Furthermore, in samples of unloaded failing human hearts, markers of autophagy were decreased whereas indicators of apoptosis were found to be increased⁸. An analysis of unloaded failing rat hearts attributed increased cell death to prolonged unloading⁹. Finally, unloading of normal hearts revealed increases in markers of cell death and protein degradation with time^{10, 11}.

Atrophy-induced apoptosis may well have implications regarding possible recovery of cardiac contractile function, as it is one of the factors that potentially counterbalance beneficial unloading effects. The time frame of atrophic remodelling processes in unloaded hearts remains poorly characterized. Also, especially in unloaded settings of human disease, the distinction between functional recovery and possible adverse consequences of ongoing atrophy can probably never be made.

The aim of our study was to identify the kinetics of load-dependent regulation of myocardial composition over time in a pre-clinical model in order to identify targets or strategies that potentially enhance myocardial recovery. A series of genes summarizing several aspects of cellular homeostasis and remodeling have been evaluated over a prolonged period of unloading. Apoptosis was further investigated at the protein level.

2. Methods

2.1 Animals

Male Lewis rats (~250g) were used as donors and recipients for heterotopic transplantation (HTX). All animals received care in accordance with Swiss Animal Protection Law.

2.2 Heterotopic transplantation/Tissue sampling

The HTX model was used as previously described⁵. Procedures were carried out under inhalative isoflurane anaesthesia (2%). Briefly, isogenic infrarenal transplantation was achieved by anastomosing the ascending aorta of the donor organ end-to-side to the abdominal aorta of the recipient, within an ischemic time of 20 minutes. Coronary artery flow antegradely perfuses the myocardium whereas the cavities remain unloaded in the

spontaneously beating graft (~300/min). The pulmonary artery of the transplant is anastomosed end-to-side to the inferior vena cava. At indicated time points, animals were sacrificed and both transplanted and native hearts (Control) were excised, then one segment was embedded in cryopreservative (TissueTec, Sakura, USA) and a second stored for RNA extraction. Separate series of hearts were transplanted for 3, 30 and 60 days for protein analyses.

2.4 Gene Expression Analysis

Total RNA was prepared from left ventricular myocardium using Qiagen RNEasy kit (all Qiagen, Hilden, Germany). Quantitative real-time PCR was performed (n=5/group) using TaqMan® Array Custom Micro Fluidic Card system (Applied Biosystems, Foster City, USA). Gene expression was assessed using the comparative $\Delta\Delta C_T$ method. All 47 transcripts (Appendix A) were analyzed. The geometric mean of the CT obtained for tubulin, β -actin, and HPRT1 were used to normalize gene expression.

2.5 Protein Analysis

LV tissue was homogenized, fractionated by 4–15% SDS-PAGE, then transferred on a PVDF membrane (Millipore). Immunoanalysis was performed (n=4–5/group) with rabbit polyclonal antibodies against bax (2772), bcl-2 (2827, both from Cell Signaling Technology, Beverly, USA), beclin-1 (sc-11427, Santa Cruz Biotechnologies, Heidelberg, Germany) and LC-3 (ab58610, abcam, Cambridge, UK). Immunoreactive products were identified using nearinfrared imaging system (Odyssey, LI-COR Biosciences, Bad Homburg, Germany).

2.6 Immunohistochemistry and quantification of apoptosis

After TUNEL staining, apoptotic nuclei were detected on 3 different sections / sample (n=7–12 samples/group) using the *in situ* cell death fluorescein detection kit (Roche Diagnostics AG) according to manufacturer's instructions. For cleaved Caspase-3 immunostainings, sections were incubated with primary antibody (9664, cell signaling, Beverly, USA) and secondary fluorescent antibody (goat anti-rabbit Alexa Fluor 488, Invitrogen, Luzern, Switzerland). Each slide was assessed for regions with apoptotic nuclei, TUNEL positive nuclei or Caspase 3-positive cardiomyocytes were normalized to the area of the section (mm^2).

2.7 20S proteasome enzyme activity

Proteasome fraction was isolated from whole myocardium samples (n=4/group) using KHPO_4 buffer according to previously published methods¹². Chymotrypsin-like enzyme activity of the 20S proteasome was measured fluorometrically ($\lambda_{\text{exc}} = 380/460\text{nm}$) by following the hydrolysis of Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin as a fluorogenic substrate (Chemicon Assay Kit, Millipore, Billerica, USA).

2.8 Statistical Analysis

Values are expressed as mean $\pm$ standard deviation (SD). Comparisons among groups were analyzed using one-way ANOVA followed by Bonferroni post-hoc test or student's t test when indicated. Statistical significance was stated at the level of $p < 0.05$.

3. Results

3.1 Myocardial unloading leads to immediate and critical loss of myocardial mass

In our experimental setting, prolonged mechanical unloading via heterotopic transplantation of the heart led to a highly reproducible ventricular weight adaptation curve characterized by two phases (Figure 1): An initial active "adaptation period" (day 0 to 15) starting

immediately after unloading, during which a rapid and dramatic loss of myocardial volume could be observed ($22.4 \pm 5.6\%$ at 3 days, $47.4 \pm 4.8\%$ at 8 days and $57.4 \pm 1.4\%$ after 15 days), followed by a “steady state period” (day 15 to 60) during which the ventricular weight loss stabilized around 65% ($61.8 \pm 2.8\%$ at 30 days, and $64.0 \pm 3.9\%$ after 60 days). The additional loss in muscle mass up to 60 days was only 6.6% after 15 days (n.s.). We sought to identify patterns in gene/protein expression that mirrored these changes.

3.2 Heterotopic transplantation induces unloading-specific gene expression changes in a timedependent manner

The analysis of selected mRNA transcripts over time (Table 1) rendered 4 distinct patterns of gene expression (Table 1, Figures 2 and 3):

3.2.1 In the first pattern, an initial period of gene expression changes immediately after the onset of unloading and was followed by a new steady state period—

In line with previously reported data, we observed that the characteristic switch of MHC isoforms α and β occurred immediately, with rapidly decreasing levels of MHC- α and increasing levels of MHC- β ($14 \pm 0.02\%$ and $450 \pm 136\%$ of baseline values at day 3, respectively). Following this initial period, MHC- α levels progressively increased, stabilizing at about 70% of control level after approximately 15 days (Figure 2A). This pattern was also found for peroxisome proliferator-activated receptor α (PPAR α), a gene involved in the regulation of myocardial metabolism that has been shown to control cardiomyocyte atrophy¹³. PPAR α appeared to be downregulated immediately after unloading was installed ($60 \pm 14\%$ at 3 days; Table 1). These data, in line with previously published short-term unloading results⁴, add further knowledge about effects of long-term unloading as the expression of this gene appeared stabilized after 15 days of unloading. Regulation of sarcomeric genes involved in the contractile function (Troponin-T, Troponin-I, SERCA2a, cardiac α -actin) also followed the pattern of rapid decline at 3 days of unloading after which they stabilized to a new steady-state level (Figure 3B,C).

3.2.2 In the second pattern, gene expression progressively increased over time with a pronunciation at the late stage of unloading—

This group consisted of genes generally reflecting extracellular matrix remodelling, such as fibroblast growth factor-2 (FGF-2), and showed a steady increase over time (Figure 3A). We also saw induction of tissue inhibitor of metalloproteinases (TIMP-1), while matrix metalloproteinases (MMP-2 and -9) were not significantly modified over time (Table 1, Appendix A). Atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) as surrogate markers of pathological myocardial stress/volume overload are implicated in hypertrophy as well as unloading-induced atrophic remodelling⁴. In our analysis, their expression continued to increase up to 60 days (Figure 3A).

3.2.3 In the third pattern, no critical change of gene expression was detectable during the initial 30 days following unloading; however a late, but dramatic, increase in gene expression was measurable at day 60—

This pattern of gene expression seemed characteristic for genes involved in apoptosis. For instance, we found a trend towards upregulation of bax and bnip-3, two critical components of the intrinsic apoptosis pathway, after 60 days of unloading (Table 1, Appendix A). The downstream Caspases 6 and 9 were also found to be significantly upregulated at day 60 (Figure 4). Interestingly, we also found the expression of the G-Protein-coupled receptor kinase-2 (GRK-2), to be significantly increased only after 60 days of unloading (Table 1), which is reflecting not only adrenergic desensitization, but has recently been shown to confer pro-apoptotic signalling¹⁴.

3.2.4 In the fourth pattern, no change was detectable during the entire 60 days of observation—This was unexpectedly the case for matrix metalloproteinases 2 and -9 (MMP-2, -9) and transforming growth factor β (TGF- β) (see Appendix 1) and may be due to the specific primers used in our commercially available assay.

3.3 Apoptotic changes occur late after unloading has been initiated

We found late expression changes of genes involved in the apoptosis process (60 days) to be mirrored at the protein level. Increased protein expression of bax led to a higher apoptotic index (bax/bcl-2 ratio) after 30 and 60 days of unloading (Figure 5). In our setting, expression of the anti-apoptotic protein bcl-2 was only decreased at the mRNA level, but not at the protein level (Figure 5). TUNEL and cleaved-Caspase-3 detection revealed patchy distribution of apoptotic cardiomyocyte nuclei throughout the myocardium, not restricted to certain areas (Figure 6). Again, quantification revealed late occurrence of apoptosis, which was significantly greater than in control hearts, but overall still at limited density compared to heart failure or ischemia-reperfusion injury (Figure 6).

3.4 Markers of autophagy and intracellular proteolysis are not increased in long-term unloaded myocardium

Previously, the induction of the ubiquitin-proteasome system has been implicated in muscle protein degradation after unloading as a major pathway¹⁵. Gene expression of Cathepsin B, a lysosomal protease known to degrade myofibrillar proteins and contribute to protein turnover, but also to apoptosis in the heart was increased 60% ($p=0.026$) (Table 1). Chymotrypsin-like activity of the 20S proteasome was measured fluorometrically and revealed an early, but transient increase in unloaded myocardium after 3 days, which dropped below control levels after 60 days of unloading (Figure 7), implying that a lack of proteolysis and protein degradation could contribute to the increase in apoptosis at late time points. Beclin-1 and LC3 isoform protein expression were unaltered with regard to control levels, suggesting that autophagy is not majorly implicated in the remodelling of normal unloaded myocardium in our setting (Appendix B).

4. Discussion

We present a non-biased examination of the dynamic gene regulation that occurs in hemodynamically unloaded normal ventricular myocardium. Serial analysis of indicators of myocardial composition and function over a 60-day period led to the identification of two phases of atrophic remodeling and, at a molecular level, to the characterization of the kinetics of myocardial adaptation. The existence and impact of unloading-related apoptosis in normal myocardium has been a matter of debate. We demonstrate increased apoptotic cell death in unloaded myocardium in a time-dependent manner and at a low level, and this is shown separately from the impact of concomitant chronic injury to the heart.

We, and others, have provided ample evidence for the atrophy induced by unloading^{4, 5}. In our hands, there was no significant further loss of myocardial mass after 15 days, suggesting the stabilization of unloading-induced atrophy, which was taken into account when determining the time points for analysis. The distinction of two phases of atrophic adaptation, first rapid decline in myocardial volume (adaptation phase), and then stabilization or maintenance of myocardial mass, were reflected in the patterns we identified for specific functional groups of genes. Thus, we may hypothesize that adaptive changes that mirror these phases are physiological, whereas late increases, for instance of indicators of pathological remodeling, could be detrimental.

Unloading of the heart induces signature transcriptional changes, some of which are similar to those observed in cardiac hypertrophy, such as activation of the fetal gene program. In line with previous reports, we show the rapid adaptation of sarcomeric (MHC α/β , Troponin-T/I-C, α -actinin), calcium-cycling (SERCA2a, RyR) and metabolic (PPAR- $\alpha/-\gamma$, mCPT-1) genes in response to the loss of mechanical load⁴. The distinct pattern of regulation, with immediate decline/elevation of transcript expression after 3 days followed by gradual, partial recovery of mRNA expression is a novel finding of this first time-course analysis. The switch of MHC-isoforms as well as the shift towards fetal-like energy metabolism in unloaded myocardium have been associated with improved function¹⁶. Certain increases in sarcomeric gene expression in unloaded, failing hearts have also been implicated in potential recovery mechanisms¹⁷. Our data show that the heart dynamically reacted to the loss of mechanical load, but the return to near normal levels of expression for contractile elements also revealed potential balancing, compensatory mechanisms in place. Certainly, the myocardium appears to swing into a new steady state with respect to the activity of a group of genes reflecting the contractile capacity of the heart.

We observed a different pattern of expression in genes generally reflecting pathological remodelling of the heart, such as indicators of matrix remodelling and also apoptosis. For these, there was either an immediate (TIMP-1) or continued (ANF) increase, or a late induction of gene expression (BNP, GRK2, bax, Caspase-6, Caspase-9). In the literature, studies addressing tissue remodelling lack uniformity in the manner of changes, some finding increase in already elevated levels of fibrosis and myocardial collagen content in failing unloaded hearts¹⁸, others reporting the opposite¹⁹. One study by Klotz et al. found that MMP-1 and -9 were decreased in unloaded failing hearts, while TIMP-1 levels rose excessively², which is similar to our findings. The shift of MMP-1/TIMP-1 ratio, in combination with high Angiotensin II levels then led to increased overall collagen content and myocardial stiffness².

The somewhat unexpected findings regarding apoptotic markers were corroborated by protein expression analysis, which verified a definite late apoptosis. Data reported by our group revealed the ratio of nuclei to cytoplasm to be significantly changed with ongoing atrophy in the unloaded myocardium⁵. One may speculate that our novel finding of late apoptosis in unloaded myocardium bears implications for the duration of circulatory support and potential of functional recovery, as the balance of beneficial reverse remodelling and progressing atrophy could be tipped towards detrimental processes, should apoptosis occur in the unloaded, failing myocardium at late stages.

Apoptosis and autophagy in unloaded cardiac muscle have been controversial. In one of the first studies addressing the subject in unloaded rat hearts, Schena et al described early induction of apoptosis after 3 days, which then slowly decreased toward normal levels¹⁰. This result seemed plausible, given that after the initial stress of surgery, including ischemic time, the heart remains perfused and rapidly adapts to the change in load without receiving further injury or stress. Interestingly, in this study there was no difference between partially loaded grafts and completely unloaded hearts, whereas the cytoplasmic index indicated significantly less atrophy in the partially loaded hearts. In a recent approach, rat hearts with induced dilative cardiomyopathy (DCM) were unloaded via HTX and these showed increased fibrosis and apoptosis compared to loaded DCM hearts not after 2, but after 4 weeks⁹. It was not reported whether this difference was due to an increase in apoptotic nuclei within the fibrotic region only or ubiquitously, but this remains an interesting question.

In human hearts, the question whether unloading leads to detrimental autophagy and/or apoptosis or not was recently approached in one study with matched samples of failing

hearts before and after implantation of an LVAD⁸. Long-term unloading resulted in decreased markers of autophagy and an increase in some markers for apoptosis such as bcl-2 and activated Caspase-3, but no statistically significant change was seen in the number of TUNEL-positive nuclei in these samples. These intriguing findings indicate that apoptosis may indeed be induced via unloading, but perhaps at a limited frequency so as not to be detected in randomly chosen samples. Previously, several investigations of apoptosis in unloaded failing hearts had reported downregulation of indicators of apoptosis at the gene and protein level⁶, as well as, for the most part, quantification of TUNEL-positive nuclei, which does not differentiate between specific types of cell death⁷.

There are limitations when interpreting our results with regard to failing hearts as well as to human myocardium: We have unloaded normal, i.e. non-failing hearts. In the clinical setting, we will not be able to separate the effect of mechanical unloading from the progression of failure and reversal of hypertrophy. Correspondingly, it has been difficult to assess the effect of unloading on cell death. This represents a gap in our knowledge that can only be estimated on the basis of experiments using this animal model. Regarding the difference between heterotopic rat heart transplantation and the reaction of human myocardium to unloading, Doenst et al. demonstrated similarities in gene expression response of failing human and healthy rat myocardium independent of species differences and underlying disease, rendering the HTX model relevant and allowing the assumption, that our results may be translatable to human disease²⁰. Certainly, further experiments with failing, unloaded rat hearts and tissue from human unloaded myocardium will be necessary to clarify the functional impact of apoptosis after long-term unloading.

Until now, there has been no set time-frame for reverse remodelling induced by unloading and an optimal duration for beneficial effects has not been described. Given our results, albeit their restricted relevance to failing myocardium due to the use of normal hearts in our study, we may speculate that after very long times of unloading there are detrimental effects that may be precluded by partial reloading or could be amenable to molecular therapeutic interventions.

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appendix A

GENE	Unloading	Regulation (fold of CTR)	p value
	(n=5/group)		
Fetal Genes			
MHC-alpha	↓	0.14±0.02	0.0001
MHC-beta	↑	8.0±0.32	0.002
ANF	↑	4.5±1.6	0.038
BNP	↑	2.4±1.4	0.044
Adrenergic receptor signaling			
GRK-2	↑	1.67±0.7	0.046
Metabolism			
PPAR alpha	↓	0.50±0.09	0.034
PPAR gamma	↓	0.38±0.13	0.032
Acid alpha glucosidase	↑	1.42±0.19	0.367
GPD 1 (glycerol-3-phosphate dehydrogenase)	↑	1.42±0.73	0.130
AGPAT (1-acylglycerol-3-phosphate-O-acyltransferase)	↘	0.79±0.19	0.266
IGF-1 (Insulin-like growth factor-1)	↗	1.51±0.72	0.577
Catalase	→	0.89±0.15	0.174
MCD (malonyl CoA decarboxylase)	↑	1.4±0.31	0.009
mCPT-1 (muscle Carnitine-Palmitoyl-Transferase)	↓	0.73±0.3	0.022
Apoptosis			
Bax	↗	1.53±0.31	0.296
Bnip-3	↗	1.8±0.9	0.768
Bcl-2	↓	0.68±0.09	0.046
Caspase-6	↑	1.79±0.5	0.0001
Caspase-9	↑	1.82±0.54	0.026
Tissue remodeling			
Fibroblast-growth-factor-2	↑	3.17±1.34	0.0001
Transforming-growth-factor beta	→	1.25±0.53	0.467
Connective tissue growth factor	↗	3.42±0.99	0.184
Calcium cycling			
SERCA-2a	↓	0.26±0.16	0.0001
Ryanodine Receptor	↓	0.61±0.46	0.342
Phospholamban	→	1.1±0.38	0.143
Muscle filament			
Cardiac alpha actin	↓	0.37±0.1	0.004

GENE	Unloading	Regulation (fold of CTR)	p value
Sarcomeric alpha actin	→	1.19±0.7	0.866
Troponin I	↑	1.71±0.3	0.009
Troponin T	↓	0.38±0.2	0.001
Troponin C	↑	2.4±0.47	0.001
Tropomyosin	↘	0.77±0.26	0.261
Cellular differentiation/turnover			
Cathepsin B	↑	1.6±0.49	0.026
Annexin A3	↘	0.59±0.09	0.126
HIF-1a	→	0.84±0.17	0.755
Smooth muscle actin	→	0.69±0.13	0.923
Matrix remodeling			
Matrix-metalloproteinase-2	→	1.27±0.74	0.552
Matrix-metalloproteinase-9	→	1.11±0.13	0.765
TIMP 1 (tissue inhibitor of metalloproteinases)	↑	4.0±3.3	0.022
TIMP 2 (tissue inhibitor of metalloproteinases)	→	1.48±0.49	0.215
Pro-alpha (III) collagen	↗	1.72±1.24	0.681
Fibronectin	↗	2.24±0.4	0.209
Osteonectin	→	1.03±0.58	0.353
Housekeeping genes			
18S	↑	2.01±0.45	0.011
GAPDH (Glycerinaldehyd-3-phosphat dehydrogenase)	↘	0.52±0.27	0.069
Alpha-tubulin	→	0.72±0.07	0.466
Beta actin	→	0.81±0.18	0.512
HPRT (hypoxanthinphosphoribosyltransferase)	↗	1.48±0.78	0.343

Appendix B

Figure 1.

Time-dependent decrease of left ventricular volume signifies the immediate atrophy occurring in the unloaded heart, which is accentuated in the first 2 weeks and thereafter stabilizes with duration of unloading (n=4–13/group, *p<0.05 compared to implantation).

Figure 2.

Gene expression of MHC isoforms α and β (grey lines), compared to housekeeping gene levels (exemplary tubulin- α , black lines), with time of unloading exhibit the characteristic switch from adult isoform, MHC- α , which is decreased, to upregulation of the β isoform. This is known as part of the fetal gene program (n=4–6/group, *p<0.05 compared to Control (native hearts)).

Figure 3.

A. Gene expression of markers of pathological myocardial remodelling revealed an increase, which was accentuated at the latest time point, 60 days after HTX (tubulin- α : black line, ANF: broken grey line, FGF-2: broken, dotted grey line), * $p < 0.05$ vs CTR. B. Effect of long-term unloading on indicators of contractile function revealed a pattern similar to the one exhibited by MHC- α mRNA expression. Alpha-actinin (broken grey line) and Troponin T (dotted grey line) showed an early decrease followed by recovery of expression to normal levels over time. The same pattern as for MHC- α (Figure 2) occurred in gene expression profile of SERCA2a, exemplary for calcium cycling (C) (* $p < 0.05$ vs CTR).

Figure 4.

Markers indicative of active detrimental effects occurring in mechanically unloaded hearts were unchanged until 30 days of unloading compared to housekeeping gene levels (tubulin- α : black line), but there was an accentuated increase after long-term (60 days) in the pro-apoptotic markers bax and Caspase-6 and -9 (please see Table 1 for detailed description). At the same time, bcl-2 gene expression was depressed (Table 1).

Figure 5.

Pro-apoptotic factor bax was increased at the protein level at late time-points, leading to an increased apoptotic index (bax/bcl-2 ratio) after 30 and 60 days of unloading (n=5/group, *p<0.05 compared to CTR)

Figure 6.

In line with the above shown data, caspase-positive cells were almost exclusively found in the hearts unloaded for a long time period. Cleaved Caspase staining could be found ubiquitously and was not confined to any specific area in the heart. Similarly, detection of TUNEL-positive nuclei was significantly increased at 30 and 60 days (n=5/group, 3 sections analysed per heart, *p<0.05 compared to CTR).

Figure 7.

The rate of protein degradation was increased after 3 days of unloading, but sunk below control levels after 60 days of unloading (n=5/group, *p<0.05 compared to CTR).

Table 1

Significantly regulated genes

Category	Gene		3d	8d	15d	30d	60d
Pathologic remodeling	ANF	↗	4.5±1.51*	2.6±1.5	2.54±1.48	2.0±1.3	3.8±2.1*
	GRK-2	↗	1.01±0.73	0.9±0.8	0.9±0.31	0.9±0.35	1.7±0.7*
Fetal Gene Program	MHC-α	↗	0.13±0.02*	0.4±0.13*	0.64±0.13*	0.6±0.04*	0.7±0.1*
	MHC-β	↗	4.5±0.86*	5.63±2.01*	5.09±3.69*	3.4±1.14	8.0±0.3*
Apoptosis	Bcl-2	↗	1.0±0.43	0.7±0.1*	1.06±0.13	0.9±0.24	1.2±0.04
	Caspase-6	↗	0.83±0.17	0.92±0.20	1.04±0.27	0.81±0.14	1.8±0.50*
	Caspase-9	↗	0.83±0.20	1.0±0.30	1.14±0.6	0.94±0.2	1.8±0.54*
	FGF-2	↗	0.77±0.09	1.24±0.13	1.56±0.6	1.24±0.35	3.2±1.34*
Tissue remodeling	TIMP-1	↗	4.0±3.33*	2.0±0.60	1.05±0.62	1.43±0.6	1.0±0.23
	Cathepsin-B	↗	1.6±0.5*	1.25±0.34	1.3±0.1	1.5±0.14	0.96±0.3
Contractile element	α-actin	↗	0.37±0.11*	0.8±0.12*	0.95±0.07	1.0±0.16	1.0±0.3
	Trop-C	↗	2.0±0.13	1.46±0.36	1.73±0.68*	1.4±0.14	2.5±0.5*
	Trop-I	↗	0.75±0.29	1.6±0.19*	1.71±0.30*	1.4±0.1	1.4±0.6
	Trop-T	↗	0.38±0.20*	0.7±0.27*	0.68±0.08*	1.1±0.22	0.6±0.1*
Calcium cycling	SERCA-2a	↗	0.26±0.16*	0.5±0.12*	0.68±0.14*	0.7±0.06*	0.7±0.14*
Metabolism	PPAR-α	↗	0.6±0.14*	0.5±0.08*	0.93±0.28	0.8±0.27	0.9±0.24
	PPAR-γ	↗	0.38±0.84	1.6±0.13	1.94±2.31	1.11±1.0	8.6±7.8*
	MCD	↗	0.68±0.17	1.0±0.19	1.18±0.34	0.9±0.04	1.4±0.3*

Category	Gene		3d	8d	15d	30d	60d
	mCPT-1		0.73±0.31	0.84±0.15	1.03±0.11	0.86±0.10*	1.25±0.24
Ribosomal RNA	18S		1.26±0.18	1.1±0.40	1.36±0.43	1.0±0.4	2.0±0.45*