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Dynamic regulation of MEK/Erks and Akt/GSK-3β in human end-stage heart failure after left ventricular mechanical support: myocardial mechanotransduction-sensitivity as a possible molecular mechanism

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Abstract

Objective: Left ventricular assist devices (LVAD) are used to 'bridge' patients with end-stage heart failure to transplantation. After long-term LVAD support, ventricular function may partially recover, a process called 'reverse remodeling'. As several kinase-mediated signal transduction pathways have been implicated in the development of cardiac hypertrophy and failure, we examined the activities of the Erks, MEKs, Akt, GSK-3β, p70S6K, JNKs and p38 under LVAD support as well as during single myocyte strain and whole heart stretch. **Methods:** Western blotting and immunohistochemistry were performed using phospho-specific antibodies in matched samples from ten patients with end-stage heart failure before and after LVAD. Cyclic strain was performed in rat neonatal cardiac myocytes, and tensile stretch applied to Langendorff-perfused mouse hearts via a left ventricular balloon. **Results:** The activity of Erks and Akt in failing hearts dramatically decreased after LVAD support, while that of GSK-3β increased. There was an endo/epicardial gradient for Erk activity which persisted after LVAD despite the reduction of total Erk activity. TUNEL-positivity and myocyte size decreased after LVAD, but independently of changes in kinase activity. In cardiomyocytes and Langendorff-perfused mouse hearts both strain/stretch and its relief regulated the activities of Erks, Akt, and GSK-3β. **Conclusion:** Erks and Akt/GSK-3β are highly responsive to myocyte stretch in vitro and in vivo, and may be sensitive molecular parameters of 'reverse remodeling' under LVAD support.

Keywords: Heart failure; Remodeling; Mechanotransduction; Protein kinases; Apoptosis; Stretch

1. Introduction

Human heart failure is a major disease in all industrial countries and largely contributes to public health expenses. Heart transplantation is an accepted therapy if medical therapy fails but only limited donor hearts are available. Therefore, left ventricular assist devices (LVAD) are being increasingly implanted to bridge patients with terminal heart failure to transplantation. The present experience with such devices shows that in some LVAD-supported patients cardiac function may improve to the extent that the device can be removed without further need of heart transplantation [1,2]. This has suggested the existence of a

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process called ‘reverse remodeling’ of the ventricles, although the exact underlying biological mechanisms are still unknown. During LVAD-mediated ‘reverse remodeling’ mean cardiomyocyte diameter, volume, length, and width decrease [3], and the expression of genes induced in the failing heart such as the atrial and brain natriuretic peptides (ANP/BNP) [4], interleukin-6 (IL-6) [5], tumor necrosis factor-α (TNF-α) [6]), apoptosis-associated genes [7] and transcription factors such as NF-κB [8] are downregulated.

In the LVAD-supported failing human heart, only limited information is available on the kinase-regulated signal transduction pathways that generally play a role in heart hypertrophy and failure. Three signal transduction pathways have been implicated in the development of cardiac hypertrophy: the mitogen-activated protein kinases (MAPKs) with the extracellular signal-related kinases (Erks), c-Jun N-terminal protein kinases (JNKs) and p38 MAPKs subfamilies [9]; the Ca^{2+}/calmodulin-activated protein kinase (CaM kinase) and phosphatase (calcineurin) [10] and, more recently, the protein kinase B/Akt and its downstream target glycogen synthase kinase-3β (GSK-3β) [11,12]. In addition, all three signaling pathways have been shown to be activated in human heart failure [13,14]. While the Erks are powerfully activated by hypertrophic agonists such as phenylephrine, angiotensin II and endothelin-1 via G-protein coupled receptors [9], the JNKs and p38-MAPKs are activated by potentially cytotoxic cellular stresses and seem to play a role in cardiomyocyte apoptosis [15]. In contrast, Akt is a powerful anti-apoptotic kinase in a number of experimental systems including cardiomyocytes [16,17], and mediates some of its biological functions via inhibitory phosphorylation of GSK-3β, an essential negative regulator of cardiac hypertrophy [18]. The second signaling pathway besides the Erks which affects the cellular growth response in a variety of biological systems [19] is the phosphatidylinositol-3-OH kinase (PI3K), which acts through Akt and p70S6 kinase (p70S6K). The latter is a key factor in angiotensin II receptor type 2-mediated cardiac hypertrophy [20] and has not yet been studied in failing human hearts under mechanical support.

In this study, we examined the activity of the mitogen-activated protein kinase kinases (MEKs), Erks, Akt, GSK-3β, p70S6K, JNKs and p38 in end-stage heart failure before and after mechanical support, and provide evidence that the MEK/Erks and Akt/GSK-3β signaling pathways are dynamically regulated after LVAD. As we find these kinases to be highly responsive to alterations of both cyclic strain and stretch in cultured rat cardiomyocytes and Langendorff-perfused mouse hearts, respectively, we suggest that they are dynamically regulated by cardiac mechanotransduction-sensing mechanisms responsive to the changes in volume and pressure overload mediated by LVAD support.

2. Methods

2.1. Patient material

Paired transmural myocardial tissue from ten patients was sampled from the left ventricular apex at the time of LVAD implantation and removal prior to transplantation, respectively. The material used in the study is routinely removed during surgery (both during LVAD implantation and at the time of heart explantation), and has been examined in an anonymous and blinded fashion. Only tissue assessed as viable by gross examination was taken to avoid sampling of scar tissue. Control tissue was taken from the left ventricular apex of unused donor hearts ($n=3$). One part of the sample was snap frozen in liquid nitrogen and the other was fixed in 4% formalin. The investigation conforms with the principles outlined in the Declaration of Helsinki.

2.2. Histology and morphometry

Formalin-fixed paraffin-embedded tissue was stained with hematoxylin/eosin and periodic acid Schiff (PAS). Cardiomyocyte diameter was measured by counting 100 cells per specimen on the cell nucleus level using the two point distance function of the analysis system (KS 300, Zeiss) as previously described [21].

2.3. Immunohistochemistry

Formalin-fixed sections were pretreated by wet autoclaving containing 0.01 M citrate buffer (pH 6.0) [22], and incubated with a polyclonal antibody against phospho-Ser473-Akt (1:200) or a monoclonal antibody against phospho-Thr202/Tyr204-Erks (1:100) overnight at 4 °C with consecutive detection with APAAP. To quantify the immunoreactivity for phospho-Erks we defined a staining score ranging from ‘0’ to ‘3’ both for cytoplasmic and nuclear staining: A complete lack of staining was scored as ‘0’, scattered positive cells (up to 10%) as ‘1’, focal moderate staining (up to 50%) as ‘2’, and more than 50% positive cells as ‘3’. The sum of the cytoplasmic and nuclear score revealed the total staining score. To test for staining gradients within the heart we separately scored the subendocardial and subepicardial regions. All analyses were performed in a blinded fashion.

2.4. In situ DNA fragmentation

We performed TUNEL-assays on frozen transmural heart sections using an ApopTaq Plus Kit (Oncor). TUNEL-positive nuclei were visualized by FITC-labeled anti-digoxigenin antibody (1:500; Roche) and counterstained with 4,6-diamidino-2-phenylindol (DAPI). The percentage of TUNEL positive cells was calculated by

counting FITC and DAPI-positive nuclei separately using an image analysis system (KS 300, Zeiss) in a minimum of 20 visual fields (a mean of 3706.5 cells ranging from 1990 to 6406 cells).

2.5. Western blotting and antibodies

Frozen myocardial samples were ground to fine powder under liquid nitrogen, solubilized in lysis buffer (in mM: Tris/HCl 50, pH 7.4; NaCl 250; EDTA 5; NaF 50; 0.5 Na_3VO_4, β-glycerophosphate 10; 0.5% NP-40, 10% glycerol, 5 μg/ml leupeptin and aprotinin) and sonicated five times for 4 s (VibraCell 72434, Bioblock Scientific). After centrifugation (2 min, 14 000×*g* at 4 °C), total protein (which includes quantitatively both cytosolic and nuclear proteins) was measured using the BCA protein assay (Pierce). Fifty micrograms of protein were separated on 10% SDS–PAGE, transferred to Immobilon membranes (Millipore), immunoblotted and visualized by enhanced chemiluminescence (Amersham). The antibody to calsequestrin was a kind gift from the Department of Pharmacology, University of Münster. All phospho-specific polyclonal antibodies were from Cell Signaling (9271, 9101, 9211, 9251, 9336, 9204) except for the anti-phospho MEK-1/2 (444945; Calbiochem).

2.6. Cell culture and cyclic stretch

Rat neonatal cardiomyocytes were isolated from the hearts of 1–2-day-old Sprague–Dawley rats using a neonatal cardiomyocyte isolation system (Worthington Biochemical Corp.) and plated on collagen type I-coated BioFlex six-well culture plates (Flexcell International Corp.) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2% horse serum, glutamine, and penicillin/streptomycin. After 48 h, myocytes were stretched (10% elongation; 60 cycles/min) using a computerized Flexcell Strain Unit for the indicated periods of time. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.7. Continuous tensile wall stretch in Langendorff-perfused mouse hearts

Isolated hearts of 3-month-old CD1 male mice were excised via median thoracotomy and perfused on a vertical Langendorff apparatus (Hugo Sachs Harvard Apparatus) using a modified 37 °C warm oxygenated Krebs–Henseleit solution (in mM: NaCl 118; $NaHCO_3$ 24.88; KH_2PO 41.2; glucose 5.55; Na-pyruvate 2; $MgSO_4$ 0.83; $CaCl_2$ 1.8; KCl 4.7) at a constant perfusion pressure of 90–100 mmHg and a flow rate of 4±1 ml/min [23]. To simulate continuous stretch, a custom-made thin latex balloon was inserted into the left ventricular cavity via the mitral valve and inflated to yield a left ventricular end-diastolic pressure of 30 mmHg for the indicated times (left ventricular pressure was continuously recorded using a pressure transducer; Hugo Sachs Harvard Apparatus). The hearts of age- and sex-matched control animals perfused without inflation of the balloon for identical periods of time served as controls.

2.8. Statistical analysis

All patient data were calculated using SPSS 10.0 for Windows. Data before and after LVAD support from the same patient were tested for significance with the Wilcoxon matched-pair test. In the box plots used, the line inside the rectangle indicates the median, and its upper and lower boundaries the upper and lower quartile, respectively. The two lines indicate data lying between the rectangle and the last values that are not outliers. Outliers are defined as values outside the thresholds, which are one step (1.5 times the interquartile range) away from the lower or upper quartile. All outliers were included in the statistics. Data on the kinase response to stretch in isolated mouse hearts was statistically analyzed using Student's *t*-test.

3. Results

3.1. Patients under LVAD support

Paired tissue from ten patients suffering from terminal heart failure (five with dilated and five with ischemic cardiomyopathy) before and after the implantation of a LVAD was gathered for analysis. The patients' characteristics are summarized in Table 1: the mean age at the time of LVAD implantation was 40.5 years (median 42.5, range 18–51); the mean duration of LVAD support was 222.4 days (median 228, range 146–295); the mean left ventricular end-diastolic diameter (LVEDD) decreased significantly ($P<0.05$) from 72.8 mm (median 71.5, range 62.0–82.0 mm) before LVAD to 65.1 mm (median 64.0, range 55.0–90.0 mm) at the end of LVAD support as estimated by echocardiography, and, accordingly, mitral insufficiency decreased. The main difference in medication between pre- and post-LVAD was the increase in beta-blocker recipients (six patients before, ten patients after LVAD) and the reduction of catecholamines (six patients before, one patient after LVAD) (Table 1).

3.2. Inactivation of MEK/Erks and Akt signal transduction pathways and activation of GSK-3β under LVAD support as measured by kinase phosphorylation

To test for LVAD-associated changes in the activation profile of kinases involved in signaling during heart failure, we examined the phosphorylation states of the

Table 1
Patient characteristics

Patient	Gender	Age (years)	Diagnosis	LVAD duration (days)	Medication before LVAD	Medication after LVAD	Difference after LVAD	
							LVEDD (mm)	MI (score)
1	Male	51	IHD	229	Dob, ACE, Dg, AC	BB, Du, S, AC	−10	−1
2	Male	51	DCM	201	Dob, BB, S, AC	BB, S, AC	n.a.	n.a.
3	Male	43	DCM	150	Dob, ACE, Dg, BB, AC	ACE, BB, AC	n.a.	n.a.
4	Male	27	DCM	228	Dob, ACE, Du, AC	ACE, BB, AC	−25	−2
5	Male	40	DCM	249	Dob, ACE, Dg, BB, Du, S	ACE, Dg, BB, Du, S, AC	8	0
6	Male	42	IHD	175	ACE, Dg, BB, Du, S, AC	ACE, Dg, BB, Du, AC	−5	0
7	Male	45	IHD	146	N	ACE, Dg, BB, N, AC	−1	−1
8	Male	18	DCM	286	ACE, Dg, BB, Du, S, AC	ACE, Dg, BB, Du, AC	−11	−2
9	Male	41	IHD	265	Dob, BB, N	ACE, Dg, BB, Du, S, N, AC	−11	−2
10	Male	51	IHD	295	ACE, Du, N, AC	Dob, ACE, Dg, BB, AC	−6	0

IHD, ischemic heart disease; DCM, dilative cardiomyopathy; LVEDD, left ventricular end-diastolic diameter; MI, mitral insufficiency expressed as score; n.a., not available; Dob, dobutamine; ACE, angiotensin-converting enzyme inhibitor; N, nitrate; Dg, digoxin; BB, beta-blocker; Du, diuretic; S, spironolactone; AC, anticoagulation.

Erks, MEKs, Akt, GSK-3β, p70S6K, JNKs and p38 in paired tissue from ten patients before and after LVAD support. We observed a dramatic decrease in the amounts of the dually phosphorylated (Thr202/Tyr204), active forms of Erk-1 and Erk-2 after LVAD support as determined by Western blot analysis using phospho-specific antibodies (Fig. 1A). Calsequestrin levels which were used as a loading marker for cardiomyocytes were similar

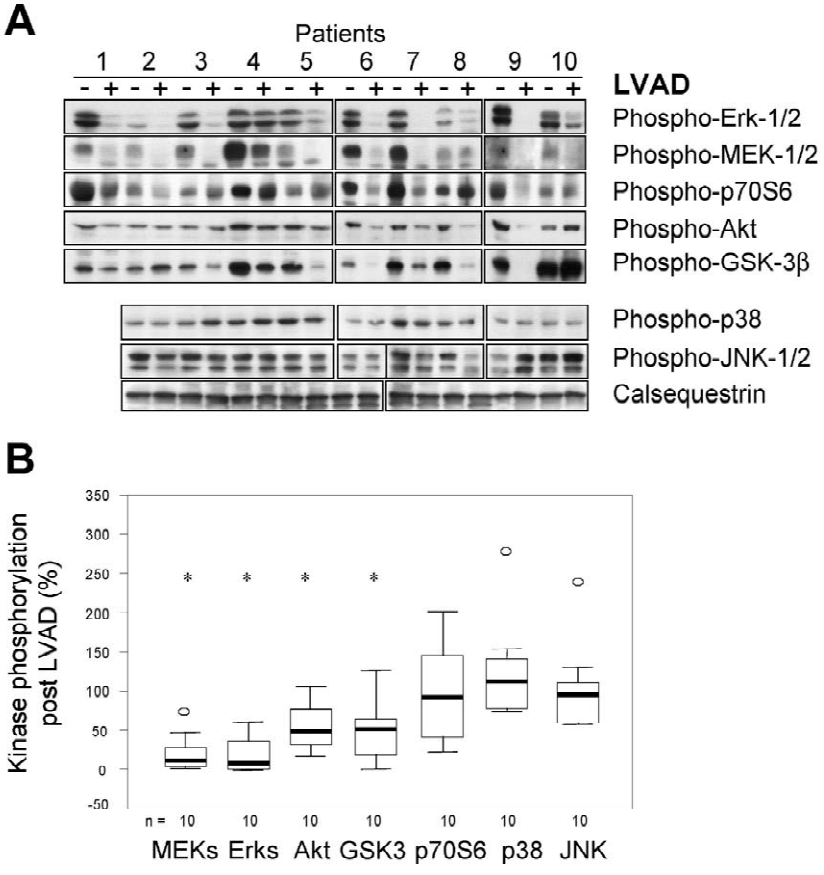

Fig. 1. (A) Western blot analysis of kinase phosphorylation profiles before and after LVAD support in paired tissue samples from ten patients ('−', prior to LVAD support; '+', after LVAD). (B) Densitometric quantification of the relative changes in kinase phosphorylation after LVAD support in percent compared to the pre-LVAD value (100%). Asterisks indicate a significant decrease in phosphorylation ($P<0.05$), open circles indicate outliers. Box plots contain the median (black bold horizontal bar) as well as the 25th and 75th percentile (fine horizontal bars).

among all samples (Fig. 1A). The total amounts of the kinases were similar in all samples (data not shown). To quantitate the changes in kinase activity, we calculated the ratio between the densitometric values of kinase phosphorylation before LVAD (set as 100%) and after LVAD for each patient (Fig. 1B): Erk phosphorylation decreased significantly ($P<0.005$) after LVAD to a mean level of 19.5% (median 7.65, range 0.03–60.59%). MEK-1/2, the kinases that activate the Erks, also showed a significant ($P<0.005$) decrease in phosphorylation after LVAD to a mean level of 24.1% (median 13.3, range 2.44–74.3%) (Fig. 1A,B) as measured using phospho-specific antibodies to Ser217/Ser221. Furthermore, there was a significant correlation between the decrease in Erk and MEK phosphorylation ($r=0.763$, $P<0.01$).

As the Akt signaling pathway has been shown to be activated in heart failure [13], we examined the phosphorylation state of Akt and its downstream target GSK-3β, a kinase inactivated by Akt-mediated phosphorylation, using antibodies to phospho-Ser473-Akt and phospho-Ser9-GSK-3β (Fig. 1A). Densitometric quantitation revealed a significant decrease in Akt- and GSK-3β-phosphorylation after LVAD support ($P<0.007$ and $P<0.013$, respectively) with a decrease in phospho-Akt to a mean level of 48.5% (median 44.4%, range 17.36–105.5%) and GSK-3β to a mean level of 43.3% (median 34.1%, range 0.29–118.0%) (Fig. 1B). There was a significant correlation between Akt and GSK-3β changes ($r=0.88$, $P<0.001$). These data suggest an inactivation of Akt and activation of GSK-3β, respectively, under LVAD support.

3.3. TUNEL-positive cells decrease after LVAD independently of changes in Akt or Erks

As both Akt and Erks are protective factors against apoptosis in a number of experimental systems, and apoptosis of cardiomyocytes has been implicated in the pathogenesis of heart failure, we tested whether there is a decrease in TUNEL-positivity after LVAD support, and whether it correlates with the individual changes in kinase activity we observe in each patient. We detected a significant decrease of TUNEL-positive cells from 0.86% before LVAD support (median 0.55, range 0.34–2.78%) to 0.28% after LVAD support (median 0.26, range 0.04–0.92%) in all eight examined patients ($P<0.012$) (Fig. 2A). However, there was no significant correlation between the decrease of Akt, Erks or GSK-3β phosphorylation with the reduction in TUNEL-positivity.

3.4. Alterations in other kinase activities under LVAD support

A different member of the PI3K signal transduction pathway besides Akt, p70S6K, showed a distinct regulation under LVAD: in five out of ten patients, there was a dramatic decrease in the phosphorylation of Thr421/Ser424 (which positively regulates kinase activity), while the remaining five patients showed no major changes (Fig. 1A). Interestingly, the reduction in kinase phosphorylation was observed in all patients with high p70S6K phosphorylation before LVAD, while no reduction was observed in the subgroup of patients with low p70S6K phosphorylation before LVAD. An isoform of p70S6K, p85S6 kinase, was regulated identically (data not shown). As p70S6K has been implicated in the regulation of cell growth and protein translation [24] as well as in the cardiac hypertrophy mediated by the angiotensin II receptor type 2 [20], we tested if there is a correlation between the activation state of p70S6K and any changes in myocyte size under LVAD. The mean longitudinal cell diameter decreased significantly ($P<0.005$) from 23.1 μm (median 22.4, range 18.3–26.8 μm) to 19.5 μm (median 18.7, range 15.7–23.6 μm) (Fig. 2B). However, there was no correlation of cell diameter reduction and phosphorylation of p70S6K (data not shown). In contrast to the five kinases described above, the phosphorylation state of JNK-1, JNK-2, and p38 was not regulated under LVAD support (Fig. 1A,B). Accordingly, there was no change in the phos-

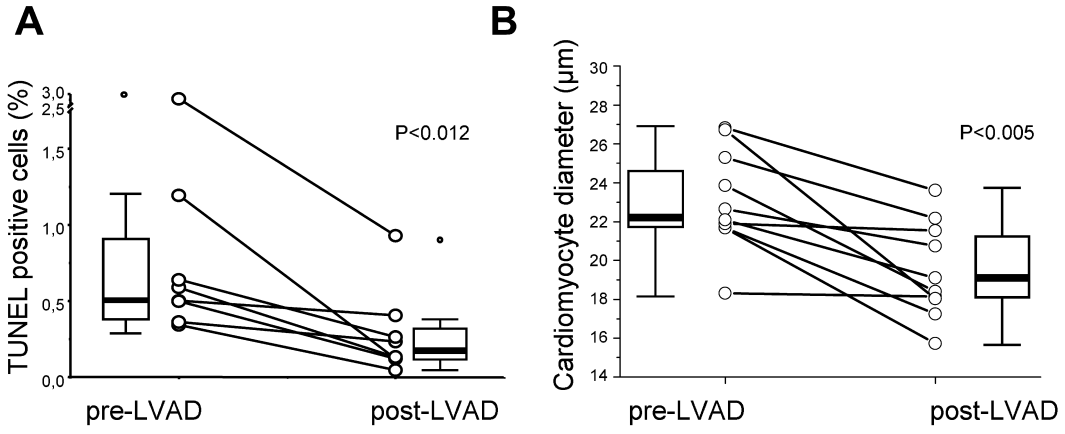

Fig. 2. (A) Analysis of TUNEL-positive cells (in percent) before and after LVAD support in eight patients. Open circles indicate outliers. (B) Effect of LVAD support on cardiomyocyte diameter in the left ventricle in ten patients.

phorylated form of the JNK substrate c-jun (data not shown).

3.5. Intracardiac gradients for Erk activity under LVAD support

To test for intracardiac gradients of kinase activity, we determined the cellular and spatial localization of the phosphorylated forms of Erks and Akt in failing hearts before and after LVAD support as well as in donor hearts using immunohistochemistry. While donor hearts showed no immunoreactivity for phospho-Erks in cardiomyocytes, there was a substantial number of phospho-Erks positive cardiomyocytes in failing hearts prior to LVAD implantation (Fig. 3A). The signal was localized both in the nucleus and, to a lesser extent, the cytoplasm. After LVAD support, the number of phospho-Erks immunopositive cells decreased substantially (Fig. 3A). Immunostaining for phospho-Akt was absent in donor hearts, and prominent in failing hearts where it localized mainly in the cardiomyocyte nuclei (Fig. 3A). After LVAD support, the immunostaining for phospho-Akt decreased substantially (Fig. 3A). These data confirmed at the cellular level the regulation of both kinases seen on Western blots.

To test for intracardiac myocyte-specific gradients of phospho-Erks, we defined a semi-quantitative cardiomyocyte staining score ranging from '0' to '3' for cytoplasmic and nuclear staining each, and separately analyzed the subendocardial and subepicardial regions. Prior to LVAD support, the subendocardial region had an approximately twofold higher mean staining score compared to the subepicardial region ($P<0.033$): 2.8, range 0–6 versus 1.3, range 0–4) (Fig. 3B). This difference was still preserved after LVAD support ($P<0.04$): mean 1.7, range 0–4) versus mean 0.6, range 0–5). The reduction of total phospho-Erks immunoreactivity was due to a significant reduction in both the subendocardial and subepicardial region: the median subendocardial score was 2.5 before and 2.0 after LVAD, respectively; the median subepicardial score was 1.0 before LVAD and 0.0 after LVAD (Fig. 3B).

3.6. The Erks and Akt signaling pathways are regulated by cyclic strain and tensile stretch in cultured rat neonatal myocytes and Langendorff-perfused mouse hearts

The reduction of Erk activation under LVAD support along with its predominant localization in the subendocardium, the region with the highest wall stress, suggested that there may be a correlation between the mechanical stretch applied to the cardiomyocyte and kinase activity. To test this, we exposed cultured rat neonatal cardiomyocytes to cyclic strain (10% elongation, frequency of 60 cycles/min) and examined kinase activities before and after cessation of strain. We observed a dramatic increase in the phosphorylation of Erks, Akt, and GSK-3β after 5 min of cyclic strain, which persisted up to 30 min and decreased thereafter ($n=4$) (Fig. 4A). After cessation of cyclic strain, there was a rapid decrease in Erk activation below basal levels with an almost complete loss of the signal after 10 min of relaxation. Interestingly, this was followed by a new activation of Erks with a maximum 30 min after relaxation (Fig. 4A). Akt and GSK-3β phosphorylation followed a similar profile both after cyclic strain and relaxation, including the second relaxation-related phosphorylation peak. However, this second peak occurred earlier than that of the Erks with a maximum 15 min after relaxation (Fig. 4A). In contrast, although there was a transient phosphorylation of p70S6K after exposure of cells to cyclic strain, we did not observe a relaxation-associated activation of the kinase.

To test whether the kinases regulated by cyclic strain in cultured cardiomyocytes are regulated by changes in wall tension in whole hearts, we distended the left ventricle of Langendorff-perfused adult mouse hearts with an inflatable balloon to yield a left ventricular end-diastolic pressure of 30 mmHg under continuous perfusion and measured kinase phosphorylation ($n=5$) (Fig. 4B). Throughout the perfusion protocol, the hearts were in sinus rhythm and generated a stable physiological systolic left ventricular pressure of 80–110 mmHg. After application of continuous wall tension for 5 min, there was a substantial activation of the Erks (2.77 ± 0.55-fold compared to control hearts perfused for the same time without stretch) (Fig. 4B,C). When wall tension was reduced by deflating the balloon for 10 min, Erk phosphorylation decreased significantly ($P<0.003$) to 1.57 ± 0.35-fold of the controls (Fig. 4B,C). Akt was also phosphorylated after 10 min of stretch (1.98 ± 0.27-fold compared to control hearts) and was significantly ($P<0.005$) dephosphorylated 10 min after cessation of stretch (1.19 ± 0.38) (Fig. 4B,C).

4. Discussion

In the present study, we demonstrated that mechanical unloading of the failing human heart through LVAD support resulted in the significant inactivation of both the MEK/Erk and Akt signal transduction pathways as well as activation of GSK-3β as measured by changes of kinase phosphorylation. The changes in kinase activity after LVAD support were dramatic (~80% MEK and Erk inactivation and 50% Akt inactivation, respectively, and 50% activation of GSK-3β) compared to the changes in clinical parameters such as LVEDD decrease. Therefore, these kinases appear to be much more sensitive molecular parameters of LVAD-mediated mechanical volume and pressure unloading compared with the alterations in gene expression in response to LVAD [3–7].

The intraindividual decrease in MEK activity after LVAD correlated with that of the Erks, while the decrease in Akt activity correlated with the increase in GSK-3β

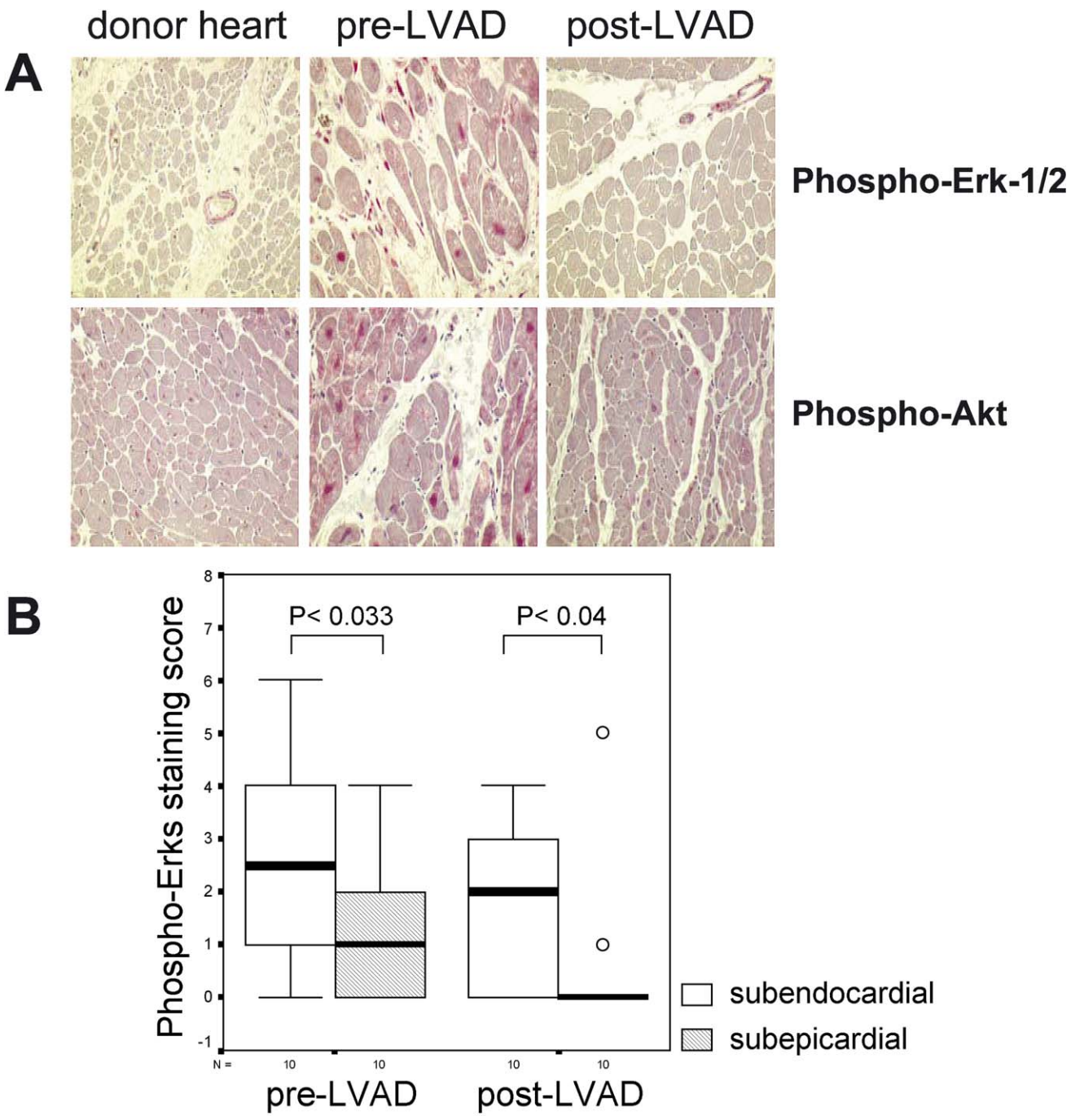

Fig. 3. (A) Immunohistochemical staining for phospho-Erks and phospho-Akt, respectively, in representative mid-myocardial sections of donor hearts ($n=3$), unsupported failing hearts (pre-LVAD) and LVAD-supported hearts (post-LVAD) ($n=10$ each) (200-fold magnification). In donor hearts, cardiomyocytes are negative, while a mainly nuclear staining is present in failing hearts which decreases substantially after LVAD. (B) Intracardiac gradients for phospho-Erks before and after LVAD support. The subendocardial and subepicardial regions were scored for phospho-Erks as described in Section 2.

activation. This suggests involvement of the respective upstream signaling pathway rather than of other pathways (as GSK-3β can be inhibited by several other kinases such as protein kinases A and C and p70S6K [12]). Our findings that GSK-3β is activated after LVAD support underlines the recently emerging evidence for its major role in inhibition of cardiac hypertrophy and development of cardiomyopathy in vitro and in vivo [12]. The MEK/Erks and Akt/GSK-3β signaling cascades execute together with CaM kinase and calcineurin the 'hypertrophic program' of the heart which is activated after stimulation of β-adrenergic and G_q-coupled receptors and results in profound changes in transcription, translation and cytoskeletal organization [9,10]. However, the MEK/Erks signaling

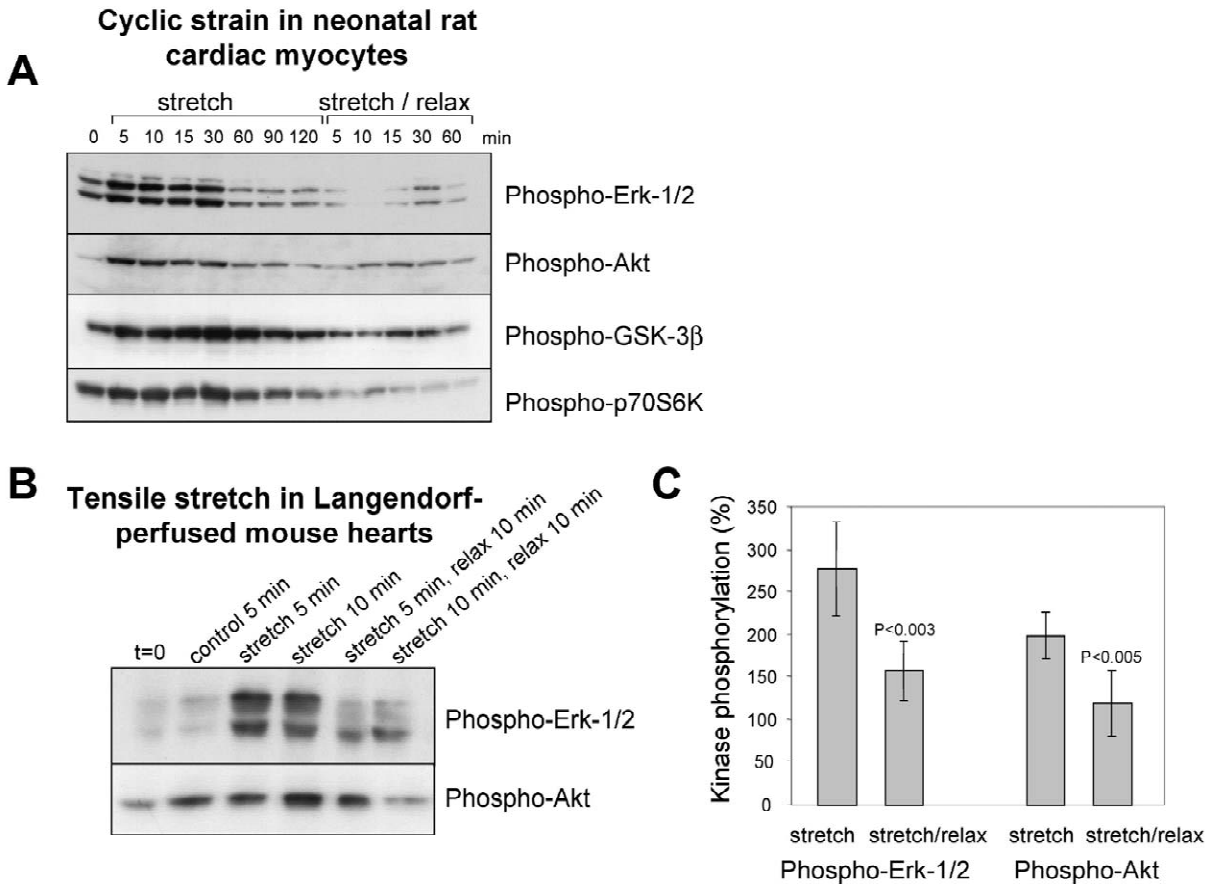

Fig. 4. (A) Both cyclic strain and relaxation activate Erks, Akt and GSK-3β in cardiomyocytes. Cultured neonatal rat cardiomyocytes were exposed to cyclic strain (10% elongation, frequency of 60 cycles/min) for the indicated times ('stretch'). Part of the cells were exposed to cyclic strain for 120 min with a subsequent relaxation for the indicated times ('stretch/relax'). Cell lysates were examined for phosphorylated Erks, Akt, GSK-3β and p70S6K by Western blot analysis ($n=4$). (B) Tensile stretch activates Erks and Akt in Langendorff-perfused mouse hearts. The left ventricle of Langendorff-perfused adult mouse hearts was distended with a balloon to yield a left ventricular end-diastolic pressure of 30 mmHg under continuous perfusion. All hearts generated a stable physiological systolic left ventricular pressure (80–110 mmHg) under sinus rhythm. The following stretch protocols were performed: (1) 5 min of stretch; (2) 10 min of stretch; (3) 5 min of stretch followed by 10 min without stretch (relax); (4) 10 min of stretch followed by 10 min without stretch (relax). Control hearts were perfused without stretch for 0 min ($t=0$) and 5 min, respectively. Left ventricular tissue was examined by Western blot analysis ($n=3$). (C): Densitometric quantification of the relative changes in kinase phosphorylation in Langendorff-perfused hearts after stretch (5 min for Erks and 10 min for Akt, respectively) as well as 10 min after cessation of stretch in percent compared to control hearts perfused for the same amounts of time ($n=5$ each). Statistics were performed using Student's t-test.

pathway promotes this program, while GSK-3β opposes it [12]. Therefore, the inactivation of MEK/Erks and the activation of GSK-3β we observed after LVAD support are in agreement with the opposing effects of the two signaling pathways in cardiac homeostasis. In contrast, neither the JNK nor the p38-mediated signaling cascades were altered under LVAD support in our study. This suggests a tight and specific regulation of kinase signaling after mechanical unloading of the human heart in vivo. A recent report described an inactivation of Erks after LVAD support as well as lack of JNK phosphorylation changes in corroboration of our study but, in contrast to our findings, an activation of p38 [25]. This may be due to a particular sensitivity of p38 to the duration of LVAD support (which differs substantially between the two studies), the inability to distinguish between different p38 isoforms as well as differences in medication.

Akt is an important anti-apoptotic kinase in a number of experimental systems [19]. In our study, there is a significant (3–4-fold) reduction of TUNEL-positive cells after LVAD support. However, we observed no statistically significant correlation between reduction in Akt activity and reduction of TUNEL-positivity. Furthermore, there was no change in phosphorylation of the pro-apoptotic forkhead transcription factor FKHR, an important target of Akt (data not shown). Another biological function of Akt is its participation together with p70S6K in the PI3K pathway, which has a profound influence on cellular growth [19]: both kinases have been implicated in the regulation of cell size in *Drosophila* and mice [26,27]. However, although we observed a marked reduction of cardiomyocyte size after LVAD support, it did not correlate with the reduction in Akt activity. We have no explanation why 50% of the patients have a high p70S6K phosphoryla-

tion prior to LVAD implantation (with a dramatic decrease thereafter) and 50% have a low one (with no changes after LVAD) (Fig. 1), and our attempts to correlate it with individual changes in cell size were unsuccessful. However, the regulation of p70S6K is complex as there is a number of other differentially phosphorylated residues important for enzyme activity besides Thr421/Ser424 [19]. Our inability to correlate the reduction in TUNEL-positivity or cell size with the changes in all kinases examined does not exclude causal relationships, as our study may not be sensitive enough to follow the complex temporal and spatial relationships between these processes.

Immunohistochemical staining for active Erks revealed an intracardiac gradient with the highest staining in the subendocardial region that, in contrast to gradients existing for other molecular parameters [8,28,29], was preserved after LVAD despite the reduction in kinase activity. One characteristic feature of the subendocardial region is that it is exposed to the highest degree of wall stress. In a number of cell types including cardiomyocytes, mechanical stretch and strain have profound effects on intracellular signaling [30–32]. Activation of Erks has been described under cyclic strain in rat cardiomyocytes, and the autocrine/paracrine action of growth factors and reactive oxygen species, respectively, has been implicated in this process [33,34]. In the present study, we extend these observations to the identification of the Erks and Akt/GSK-3β as mechanotransduction-sensitive kinases in cardiomyocytes activated not only through cyclic strain but also through its cessation. In addition, we show that the same kinases respond to acute changes in wall tension such as the simulation of an end-diastolic pressure of 30 mmHg in the whole mouse heart. Remarkably, the activation could be reversed after cessation of mechanical stretch and, in contrast to the cell culture studies, without a second, relaxation-related activation of the kinases. This suggests both similar and unique response mechanisms to stretch in isolated myocytes and the whole heart. Interestingly, a recent study using paced cardiomyocytes showed a remarkable sensitivity of Erk phosphorylation to mechanical strain dependent on the phase of the cardiac cycle, with the highest activation during systolic strain [35].

Despite the clinical observation of improvement of patients with end-stage heart failure after LVAD [2], little is known about the intracellular mechanisms involved in the reverse remodeling of the myocardium. Through the relief of volume and pressure overload, the LVAD-supported heart is exposed to relatively abrupt changes in wall tension at the cellular level. Therefore, the dramatic and uniform reduction of the MEK/Erks and Akt activities as well as the activation of GSK-3β (but not that of other kinases) argues for a high degree of specificity and sensitivity of these pathways to the pressure relief mediated by LVAD and suggests, together with the in vitro data, a mechanotransduction-sensitive component of their regulatory mechanism in vivo. The functional targets and biological functions of these kinases in the terminally failing supported heart are still unknown, but their prominent position at the crossroads between cell growth, metabolism and apoptosis makes them promising targets for future therapies.

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