Different activation of MAPKs and Akt/GSK3β after preload vs. afterload elevation

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Abstract

Aims Pressure overload (PO) and volume overload (VO) lead to concentric or eccentric hypertrophy. Previously, we could show that activation of signalling cascades differ in in vivo mouse models. Activation of these signal cascades could either be induced by intrinsic load sensing or neuro-endocrine substances like catecholamines or the renin-angiotensin-aldosterone system.

Methods and results We therefore analysed the activation of classical cardiac signal pathways [mitogen-activated protein kinases (MAPKs) (ERK, p38, and JNK) and Akt-GSK3β] in vitro of mechanical overload (ejecting heart model, rabbit and human isolated muscle strips). Selective elevation of preload in vitro increased AKT and GSK3β phosphorylation after 15 min in isolated rabbit muscles strips (AKT 49%, GSK3β 26%, P < 0.05) and in mouse ejecting hearts (AKT 51%, GSK49%, P < 0.05), whereas phosphorylation of MAPKs was not influenced by increased preload. Selective elevation of afterload revealed an increase in ERK phosphorylation in the ejecting heart (43%, P < 0.05), but not in AKT, GSK3β, and the other MAPKs. Elevation of preload and afterload in the ejecting heart induced a significant phosphorylation of ERK (95%, P < 0.001) and showed a moderate increased AKT (P = 0.14) and GSK3β (P = 0.21) phosphorylation, which did not reach significance. Preload and afterload elevation in muscles strips from human failing hearts showed neither AKT nor ERK phosphorylation changes.

Conclusions Our data show that preload activates the AKT–GSK3β and afterload the ERK pathway in vitro, indicating an intrinsic mechanism independent of endocrine signalling.

Keywords Hypertrophy; Preload; Afterload; AKT-GSK3β; MAPKs

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Introduction

Left ventricular hypertrophy is a harbinger of cardiac morbidity and mortality.1 Haemodynamic load and neurohumoral stimulation play dominant roles in the regulation of myocardial gene expression, development of cardiac hypertrophy, and consequently during heart failure (HF) progression.2,3

Haemodynamic overload can be differentiated in pressure overload (PO) or volume overload (VO). Conditions of PO with high afterload, such as aortic stenosis and hypertension, result in concentric hypertrophy, which is characterized by an increase in ventricular wall thickness and little or no chamber dilation.4,5 Conversely, conditions of VO with an increase in cardiac preload, as evident in pathological conditions such as mitral or aortal regurgitation, promote an eccentric pattern of hypertrophy. This is characterized by a relatively little increase in wall thickness and a disproportionately large increase in chamber volume.5

Several kinases have been implicated in the regulation of hypertrophy and HF. The phosphatidylinositol-3-kinases (PI3Ks) enzymes were shown to be cardioprotective through regulation of cell growth and differentiation. Binding of the PI3K to pleckstrin homology domain from 3-phosphoinositide-dependent protein kinase 1 (PDK1) leads to activation of this kinase.6 Protein kinase B (Akt) is amongst the most important downstream targets that can be activated through...
PDK1-dependent phosphorylation. Three isofoms are known for Akt, of which Akt1 and Akt2 are preferably expressed in cardiomyocytes. The Akt signalling pathway plays an important role in physiological heart growth. Once activated, Akt in turn phosphorylates and thereby inactivates glycogen synthase kinase 3β (GSK3β) that affects development of cardiac hypertrophy through derepression of different targets, for example, eIF2B, β-catenin, c-Myc, nuclear factor of activated T-cells (NFAT), and GATA4. Another important target of the Akt signalling pathway is the mammalian target of rapamycin (mTOR). Activated mTOR improves protein synthesis via the two signalling pathways p70/S6 kinase-1 and p70S6K2. Blocking mTOR prevents both pathological hypertrophy and cardiac dysfunction and may thus have beneficial therapeutic benefits.

Mitogen-activated protein kinases (MAPKs) consist of three kinases: the extracellular signal-regulating kinases (ERKs), p38, and JUN N-terminal kinases (JNKs). The activation of ERK1 and ERK2 takes place through their phosphorylation by upstream mitogen-activated protein kinase kinases (MEK) 1 and 2. Overexpression of MEK1 and MEK2 results in adaptive concentric hypertrophy of the heart, and the interaction with the calcineurin/NFAT signalling pathway was shown to promote cardiac hypertrophy. The MEK5/ERK5 signalling pathway leads to an increase in the number of cardiac sarcomeres connected in series and to eccentric dilated growth. p38 kinases and JNKs comprise the MAPK cascade and are activated by MEK3/MEK6 and MEK4/MEK7, respectively. p38 kinases and JNKs phosphorylate and activate GATA4-mediated transcription to promote pathological hypertrophy. MAPK signalling has been extensively reviewed before.

Previously, we have shown that Akt and MAPK phosphorylation are differentially regulated in preload vs. afterload. However, it is not yet clear whether the activation of these important signalling molecules is due to an endocrine receptor-mediated stimulation or due to intrinsic sensing of mechanical overload. We therefore investigated the regulation of the intracellular signalling pathways involving MAPKs and AKT/GSK3β in vitro using rabbit and human heart muscle tissue under different loading conditions, that is, preload vs. afterload.

Methods

Rabbit muscle preparation and mechanical stretch

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Female chinchilla bastard rabbits (1.5 to 2 kg, Charles River, Kisslegg, Germany) were heparinized and anaesthetized with thiopental sodium (50 mg/kg i.v.). Hearts were excised and retrograde perfused with modified Krebs–Henseleit solution as described. Right ventricular trabeculae or thin papillary muscles were dissected and mounted in culture chambers (Scientific Instruments, Heidelberg, Germany) between a force transducer and a hook connected to a micrometre drive allowing for length adjustment. The system is equipped with a servomotor with force-feedback function and allows cultivation of functionally intact multicellular muscle preparations for up to 48 h at 37°C with physiological protein turnover maintained. After [Ca2+]o was stepwise raised to 1.0 mmol/L, the Krebs–Henseleit solution was replaced with tissue culture medium at 1.25 mmol/L [Ca2+] (M-199, Invitrogen, Karlsruhe, Germany) supplemented with 20 IU/L human insulin, 0.2% (wt/vol) BSA, 70 μmol/L streptomycin, and 100 IU/mL penicillin and equilibrated with 100% O2. Preparations were allowed to stabilize for 1 h under continuous electrical stimulation (1 Hz, 3 to 5 V) and subsequently assigned to the different experimental groups. In Group 1, the isotonic group, preparations were stretched progressively over 30 s to 3 mN/mm2 resting tension, corresponding to Lmax at which isotropic tension would be maximum. In Group 2, the isotonic group, preparations were also stretched to 3 mN/mm2 resting tension and allowed to shorten isotonically from this level of resting tension. Isotonic shortening means that afterload is zero. Group 3 was unloaded; that is, preparations remained slack under otherwise identical conditions. Isometric force development or isotonic shortening was recorded continuously over 24 h at the designated loading conditions. At the end of the incubation period, muscle preparations were harvested from the culture chamber, rapidly frozen in liquid N2, and stored at −80°C.

Human myocardial tissue

All procedures performed conform to the Declaration of Helsinki and were approved by the local ethics committee. Written informed consent was received from all participants prior to inclusion in the study. Left ventricular myocardial tissue was taken from explanted hearts of patients with end-stage HF [New York Heart Association HF classification IV (NYHA IV)] undergoing heart transplantation. Detailed patient characteristics are provided in Table 1. The explanted hearts were acquired directly in the operating theatre during surgical procedures and immediately placed in cooled cardioplegic solution. Right ventricular muscles strips were dissected from the hearts and placed in the system as described below.

Human myocardial muscle strip preparation and experiments

Myocardial muscle strip preparation and experiments were performed as previously described. Calcium...
Table 1 Characteristics of patients included in the muscle strips experiments

| Characteristic                  | HF (n = 5; 0 DCM, 5 ICM) |
|--------------------------------|--------------------------|
| Male sex (%)                   | 80.0                     |
| Age (mean ± SEM, years)        | 61.4 ± 1.99              |
| EF (mean ± SEM, %)             | 19 ± 1.97                |
| CI (mean ± SEM, L/min*m²)      | 2.23 ± 0.11              |
| LA diameter (mean ± SEM, mm)   | 50.33 ± 1.91             |
| LVEDD (mean ± SEM, mm)         | 72 ± 2.73                |
| IVS (mean ± SEM, mm)           | 9.25 ± 0.41              |
| PCW (mean ± SEM, mmHg)         | 24.8 ± 1.53              |
| Ischaemic heart disease (%)    | 100                      |
| LVAD (%)                       | NA                       |
| Diabetes (%)                   | 40                       |
| ACE inhibitors (%)             | 80                       |
| Beta-blockers (%)              | 60                       |
| Diuretics (%)                  | 100                      |
| Digoxin (%)                    | 60                       |
| Catecholamines (%)             | 60                       |
| Amiodarone (%)                 | 40                       |
| AT1 receptor antagonists (%)   | 0                        |
| Aldosterone antagonists (%)    | 40                       |
| PDE inhibitors (%)             | 0                        |
| Ca²⁺-channel blockers (%)      | 0                        |
| Statin (%)                     | 40                       |

ACE, angiotensin-converting enzyme; CI, cardiac index; DCM, dilated cardiomyopathy; EF, ejection fraction; HF, end-stage heart failure; ICM, ischaemic cardiomyopathy; IVS, interventricular septum; LA, left atrial; LVAD, left ventricular assist device; LVEDD, left ventricular end-diastolic diameter; NA, not available; PCW, pulmonary capillary wedge pressure; PDE, phosphodiesterase. Values are n, mean ± SEM, or n (%).

concentration and culture medium were identical to those used in the rabbit experiments described above. The only difference was that the muscle strips were stretched to human isometric \( L_{\text{max}} \) (12 mN/mm²). Muscles strips were treated as described above.

Mouse ejecting heart

Ejecting heart studies were undertaken as previously described. Animals were anaesthetized (sodium pentobarbital; 60 mg/kg i.p.) and heparinized (1000 IU/kg i.p.) and hearts dissected into ice-cold Krebs–Henseleit buffer (KHB; mmol/L: NaCl 118, KCl 3.8, KH₂PO₄ 1.18, NaHCO₃ 25, MgSO₄ 1.19, CaCl₂ 1.25, glucose 10, and Na-pyruvate 5.0) gassed with 95% O₂ – 5% CO₂. The aorta was cannulated with a blunted 19G needle and the hearts were initially perfused in Langendorff mode with KHB at a pressure of 50 mmHg and temperature of 37.5°C. The left atrium was then cannulated (19G needle) via the atrial ear and pulmonary veins were tied off. Hearts were paced at 450 b.p.m. via a right atrial electrode. Aortic pressure was measured using a transducer. The heart was then switched to the ejecting, recirculating mode (total volume 150 mL). Preload was set to 10 cmH₂O and afterload to 30 cmH₂O and the heart left contracting for 15 min. After this period, the hearts were distributed to the following four groups with different preload and afterload (Group 1: 10 cmH₂O preload and 30 cmH₂O afterload; Group 2: 10 cmH₂O preload and 80 cmH₂O afterload; Group 3: 25 cmH₂O preload and 30 cmH₂O afterload; and Group 4: 25 cmH₂O preload and 80 cmH₂O afterload). The hearts were left for another 15 min under these conditions and then harvested, dissected, and rapidly frozen in liquid N₂ and stored at –80°C.

Western immunoblot analysis

Frozen muscle strips or pieces of the left ventricle were thawed on ice in 50 mL of homogenization buffer [1% (v/v) NP 40 (IGEPAL CA-630), 10% (v/v) glycerol, 137 mM NaCl, 20 mM Tris–HCl pH 7.4, 20 mM NaF, 1 mM Na-orthovanadate, 1 mM Na-pyrophosphate, 50 mM β-glycerophosphate, 10 mM ethylene-diamine-tetra-acetic acid (EDTA) pH 8, 1 mM ethylene glycol-bis-(2-aminoethyl)-tetra-acetic acid (EGTA) pH 7, 4 µg/mL aprotinin, 4 µg/mL leupeptin, 4 µg/mL pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and homogenized. Protein of the suspensions was determined with BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) and 20 µg of samples subjected to SDS-PAGE. Western blotting was carried out according to standard protocols. The following antibodies were used: anti-P-akt, anti-akt, anti-P-GSK3β, anti-P-p38, anti-P-ERK, anti-ERK, anti-P-JNK (New England Biolabs, Ipswich, MA, USA), anti-GAPDH (BioTrend, Cologne, Germany), anti-mouse IgG, horseradish peroxidase-linked, and anti-rabbit IgG horseradish peroxidase-linked (Amersham Biosciences, Freiburg).

For quantification, an enhanced chemo luminescence detection system (Amersham) was used according to the manufacturer’s instructions.

Statistics

Force values were transferred to tension by normalizing to the cross-sectional area of a preparation, which was calculated assuming an elliptical cross section using the formula

\[
\text{Cross-sectional area} = \frac{1}{2} \times D_1 \times D_2 \times \pi,
\]

with \( D_1 \) and \( D_2 \) representing length and thickness. Gene and protein expression was analysed using unpaired Student’s t-test with values of \( P < 0.05 \) considered statistically significant.

Results

Load-dependent phosphorylation of Akt/GSK3β and MAPKs in isolated rabbit muscles strips

Stretching of rabbit heart muscle strips under isotonic conditions (only preload) led to an increase in the phosphorylation...
of Akt (+49%, n = 9, P < 0.05, Figure 1A + B) and GSK3β (+26%, n = 9, P < 0.05, Figure 1C) compared with slack preparations. In contrast, phosphorylation of ERK (+82%, n = 8, P < 0.05, Figure 1D) was increased by isometric stretch (preload and afterload) but not by isotonic stretch. Phosphorylation of p38 (n = 8, P = n.s., Figure 1E) was not changed under both conditions.

**Load-dependent phosphorylation of Akt/GSK3β and MAPks in ejecting mouse heart**

Ejecting heart experiments with different loading conditions were performed (Figure 2A). As observed in rabbit muscle strips following isotonic stretch, an exclusive increase in preload resulted in a significantly increased Akt (+51%, n = 6,
Figure 2 Effect of 15 min increased afterload, increased preload, and increased preload and afterload compared with normal loaded (control) isolated mouse ejecting hearts on MAPK Akt and GSK3β phosphorylation. (A) Example of Western blots, (B) phosphorylation of Akt, (C) phosphorylation of GSK3β, (D) phosphorylation of ERK, (E) phosphorylation of p38, and (F) phosphorylation of JNK. *P < 0.05, **P < 0.01, ***P < 0.001.

*P < 0.05, Figure 2B) and GSK3β (+46%, n = 6, P < 0.05, Figure 2C) phosphorylation. Phosphorylation of the MAPKs, however, remained unaffected. Selective increases in afterload on the other hand induced ERK phosphorylation (+43%, n = 6, P < 0.05, Figure 2D). Combined elevation of preload and afterload (as shown for rabbit muscles strips following isometric stretch) caused increases of ERK phosphorylation vs. controls (95%, P < 0.001) and vs. hearts subjected to preload...
only (+160%, \(P < 0.001\), Figure 2D). Phosphorylation levels of Akt (+34%, \(P = 0.14\)) and GSK3β (+24%, \(P = 0.21\)) showed a tendency to be higher, but this observation did not reach statistical significance (Figure 2A + B). The phosphorylation of p38 and JNK was not changed by any of the loading conditions (Figure 2E + F).

**Load-dependent phosphorylation of MAPKs and Akt/GSK3β in human failing myocardium**

We further investigated the identified load-specific activation of Akt and ERK in human failing cardiac muscle strips (Figure 3A). Here, increase in preload (isotonic conditions) or in preload and afterload (isometric condition) did not lead to an increase in either Akt or ERK phosphorylation (Figure 3B + C).

**Discussion**

The results of the present study show that (i) preload leads to an increased phosphorylation of Akt and GSK3β; (ii) afterload induces the phosphorylation of ERK; and (iii) p38 and JNK are not phosphorylated by preload or afterload in vitro. Therefore, preload and afterload differentially affect the activation of MAPKs and Akt/GSK3β.

**Mechanisms of load-induced Akt/GSK3β and ERK activation**

Activation of MAPKs and Akt is a hallmark shown in various *in vivo* and *in vitro* afterload models, that is, the transverse aortic constriction (TAC) and the isolated perfused heart Langendorff model.\(^{21,28,29}\) Importantly, both these models also show an increase in preload and can therefore not be considered to induce pure afterload.\(^{30}\) Thus, we additionally included the ejecting heart model in this study, which allowed for selective elevation of preload or afterload, respectively. Our findings revealed that ERK is activated by afterload, whereas preload leads to activation of Akt/GSK3β. To confirm these results, we subjected isolated rabbit muscles strips to isolated preload (isotonic contraction) and to preload and afterload (isometric contraction) and found comparable results. Previous findings have demonstrated that genetic ERK activation by MEK1 overexpression leads to concentric hypertrophy.\(^{16,31}\) Also, ERK is activated in HCM (hypertrophic cardiomyopathy) models.\(^{32}\) Kehat *et al.* could show that a reduction of ERK activation *in vitro* is associated with eccentric hypertrophy.\(^{31}\) In accordance, recent findings from our group confirmed this *in vivo* using the aortocaval fistula surgery as preload model.\(^{33}\) ERK2 overexpression, however, did not affect preload-induced eccentric hypertrophy.\(^{33}\)

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*Figure 3* Effect of 15 min increased preload and increased preload and afterload compared with unloaded (slack) human failing muscles strips on ERK and Akt phosphorylation. (A) Example of Western blots, (B) phosphorylation of Akt, and (C) phosphorylation of ERK.
adaptive mechanism during VO (i.e. preload)-induced HF progression.33,34

Interestingly, p38 phosphorylation was found to be enhanced through PO and VO in vivo.20 In contrast, our in vitro data did not show an increase in p38 phosphorylation, pointing towards a neurohumoral, rather than mechanical, activation.

Location of mechanosensors

The knowledge of how mechanical stress is sensed by cardiomyocytes and transduced into intracellular signals is limited. Several mechanisms are discussed. Some authors suggest pathways that involve autocrine and/or paracrine factors released by mechanical stress such as endothelin-1 or angiotensin.35 Others have suggested pathways that are partially or completely independent from humoral or neurohumoral factors but involve cellular mechanoreceptors like stretch-activated channels,36 Na+/H+-exchanger,37 Z-disc proteins like MLP,38 or costamere proteins like melusin.39

For Akt/GSK3β, a link to the myofilament proteins melusin39 and zyxin40 has been described, indicating that preload is sensed at the level of myofilaments. In contrast, mechanical ERK activation has been linked to the focal adhesion kinase (FAK) in the costamere41 or to mechanical stretch-induced signalling of the angiotensin II type 1 receptor independent of ligand activation.42,43 This indicates that membrane molecules might be more involved in the sensing of afterload.

Human failing heart

In heart muscle strips from patients with terminal HF, we could not detect any changes in the phosphorylation of the analysed signalling molecules. This can be explained by either an already high grade of mechanoactivation and kinase phosphorylation in the failing human heart.44 Therefore, it seems likely that further high loading conditions do not translate in an additional activation of signalling pathways. We also could not detect a reduction of Akt and ERK phosphorylation in the unloaded control muscle strip. This indicated that the activation of Akt and ERK signalling persists for at least a shorter time period. Longer unloading by left ventricular assist device implantation reduces ERK as well as AKT phosphorylation in human HF.29 Therefore, mechanosensing and pathological signal activation seems to require longer times for normalization after chronic activation in human HF.

Conclusions

Preload and afterload induce different signalling in vitro. Akt/GSK3β is activated by preload, whereas ERK is activated by afterload, indicating intrinsic mechanosensing without endocrine influence. In contrast, p38 is not regulated by preload or afterload in vitro, indicating an endocrine activation in vivo. Further research is needed to identify the load-specific sensors and possible therapies.

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Limitations

The data of this study are a limited assessment of the effects of mechanical stretch on cardiac signalling pathways using ex vivo systems of rabbit and human muscle strips, subjected to stretch and working heart perfusions of mouse hearts. Also, chronic effects of mechanical overload could not be studied in our ex vivo models. This provides limited insight into how the different loading conditions lead to cardiac pathology. As previously mentioned, we presented data for phosphorylation of specific kinases in relation to GAPDH expression levels. Ideally, the total levels of each kinase and the ratio of the phosphorylation/total kinase should be shown. However, due to the limited amount of tissue, these additional Western blots were not possible. However, because of the short time of the experiments (15–30 min), a change in the total kinase levels was not to be expected.

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Conflict of interest

None declared.

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N. Hartmann et al.

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