European Journal of Heart Failure (2016) **18**, 362–371
doi:10.1002/ejhf.465

RESEARCH ARTICLE

Molecular and structural transition mechanisms in long-term volume overload

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Received 17 August 2015; revised 8 November 2015; accepted 14 November 2015; online publish-ahead-of-print 23 December 2015

Aim We have previously reported that early phase (1 week) of experimental volume overload (VO) has an adaptive phenotype while wall stress-matched pressure overload (PO) is maladaptive. Here we investigate the transition from adaptation to heart failure (HF) in long-term VO.

Methods and results FVB/N wild-type mice were subjected to VO induced by aortocaval shunt, and were followed by serial echocardiography until *in vivo* left ventricular ejection fraction was below <50% (135 ± 35 days). Heart failure was evident from increased lung and liver weight and increased mortality compared with sham. Maladaptive remodelling resulted in significantly reduced sarcomeric titin phosphorylation (causing increased sarcomeric stiffness), whereas interstitial fibrosis was not increased. This was paralleled by re-expression of the fetal gene program, activation of calcium/calmodulin-dependent protein kinase II (CaMKII), decreased protein kinase B (Akt) phosphorylation, high oxidative stress, and increased apoptosis. Consistently, development of HF and mortality were significantly aggravated in *Akt*-deficient mice.

Conclusion Transition to HF in VO is associated with decreased Akt and increased CaMKII signalling pathways together with increased oxidative stress and apoptosis. Lack of interstitial fibrosis together with sarcomeric titin hypophosphorylation indicates an increased stiffness at the sarcomeric but not matrix level in VO-induced HF (in contrast to PO). Transition to HF may result from myocyte loss and myocyte dysfunction owing to increased stiffness.

Keywords Aortocaval shunt • Volume overload • Eccentric hypertrophy • Heart failure • Akt signalling

Introduction

The adaptation of the heart to mechanical stress and the development of heart failure (HF) depend on the type of load. Two types of load can be differentiated, namely preload and afterload, or volume overload (VO) and pressure overload (PO), respectively. Preload builds up during the diastolic filling of the ventricle that stretches the cardiomyocytes. This is sensed by myofilament proteins, which adapt the recruitment of contractile units and increase cardiac performance through the Frank–Starling mechanism. The giant molecule titin is stretched during preload elevation and might therefore function as a mechanosensor. Diastolic elasticity of the heart is partly controlled by titin.[1] Both titin isoforms shift from N2B to the longer N2BA isoform and phosphorylation of the titin springs modify the elastic properties of the cardiac walls and thereby, diastolic function.[1,2] During systole, afterload acts on the contractile protein complex within cardiomyocytes to produce cardiac stroke work against vascular resistance. During ejection preload declines and titin is unloaded. Both preload and afterload influence load-dependent ion channels and intracellular ion concentrations,[3] which in turn may also influence cardiac function and gene expression.

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While pathologically increased afterload occurs in patients with aortic stenosis or arterial hypertension, an increase in preload occurs mainly in patients with mitral or aortic regurgitation. Ultimately, both types of overload may result in HF. Previously, we could show that experimental VO has an adaptive phenotype early (1 week) after surgery, while induced PO has an early maladaptive phenotype with rapid deterioration into HF, although load elevation was matched to yield the same stress–time integral in both models.[4] While the mechanisms of afterload-induced HF are widely studied, the mechanisms behind the transition to HF from adaptation in VO are poorly understood.

We could also show that the serine/threonine kinase protein kinase B (Akt) was more phosphorylated in the VO model induced by aortocaval shunt, compared with PO. This kinase is involved in various cellular processes,[5] and the role of Akt in the heart has been studied in transgenic mice. It has been associated with a favourable hypertrophy of the heart, as cardiac specific overexpression of a constitutively active form of Akt, the E40K mutant, led to an 'athlete's heart' phenotype.[6] Moreover, the *Akt1*$^{-/-}$ mice were found to be resistant to swimming training-induced cardiac hypertrophy.[7] In contrast, Akt signalling also appears to participate in pathological heart growth.[8] Mice expressing Akt1 or Akt3 in the heart showed an increased heart size, interstitial fibrosis and cardiac dysfunction.[9,10] The duration of Akt activation seems to be important, as studies with inducible cardiac-specific Akt1 transgenic mice have shown that short-term activation leads to hypertrophy with preserved contractile function, whereas long-term Akt1 expression results in excessive cardiac hypertrophy associated with pathological remodelling via an imbalance of cardiac vs. vessel growth, which is induced by a deficiency in vascular endothelial growth factor-mediated angiogenesis.[11]

In this report we wanted to study the mechanisms of late HF development in chronic experimental VO, and we also aimed to address the hypothesis that Akt is important for early adaptation to preload.

Materials and methods

An expanded Methods section is available in the Supplementary material online (*Methods S1*).

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1985) and was performed in accordance with the ethical standards laid down in the Declaration of Helsinki, 1964.

Results

Model characterization

We induced VO in mice by aortocaval shunt operation and performed serial echocardiography in these mice. Eccentric cardiac hypertrophy developed early (see the Supplementary material online, *Figure S1*), and hearts were harvested when the ejection faction (EF) was reduced below <50% (mean: 135 ± 35 days after operation). The hearts of these chronic VO mice showed an increased left- and right- ventricular weight-to-tibia length (LVW/TL and RVW/TL, respectively) ratio compared with sham-operated hearts (*Figure 1A*), a 42% greater dilatation, and increased wall thickness and left ventricular (LV) chamber dimensions (*Figure 1B,C* and Supplementary material online, *Table S1*). Survival curves were analysed after 20 weeks of shunt/sham surgery. No deaths occurred after sham surgery. However, shunt surgery resulted in a mortality rate of 20% (*Figure 1D*). Mice euthanized 20 weeks after surgery exhibited significantly increased lung weight-to-tibia length (LuW/TL) and liver weight-to-tibia length (LiW/TL) ratio consistent with congestive HF as a likely cause of the increased mortality in the volume-overloaded mice (*Figure 1E*).

Cardiac phenotype of chronic volume overload

As expected, single cardiomyocyte surface area was increased in VO by ≈ 30% (*Figure 2A,B*). Cell death, estimated by the ratio of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive to total nuclei, was significantly increased by ≈ 90% in chronic VO hearts compared with sham hearts (*Figure 2C*). In addition, the level of the anti-apoptotic protein Bcl-2 was markedly decreased in VO and the ratio of BAX/Bcl-2 was significantly increased, consistent with increased apoptotic cell death signalling (Supplementary material online, *Figure S2A*). As a possible cause of apoptosis, we investigated whether VO induced myocardial oxidative stress. Indeed, myocardial superoxide (O_2^-) production assessed by dihydroethidium (DHE) showed an increase in shunt versus sham hearts (*Figure 2D*). Nitric oxide (NO) interacts with O_2^- to form oxidant peroxynitrite ($ONOO^-$), a potent oxidant whose presence may be indirectly reflected by an increase in nitrotyrosine (NT) formation. Nitrotyrosine immunostaining was increased substantially in shunt hearts (see the Supplementary material online, *Figure S2B*).

Capillary density was visualized by immunostaining for CD31 as a marker for endothelial cells. The ratio of CD31-positive to total cells did not change in VO compared with that in sham hearts (*Figure 2E*). Maintained capillary density in chronic VO was paralleled with maintained expression of vascular endothelial growth factor (VEGF-A) and CD31 (Supplementary material online, *Figure S2C*). Interestingly, the degree of fibrosis measured by trichrome staining showed only a tendency to be elevated in shunt vs. sham mice (*Figure 2F*). Expression of Collagen3α1 (the main constituents of the cardiac extracellular matrix) and alpha-smooth muscle actin (αSMA) (a primary marker of fibroblast-to-myofibroblast conversion) were also not changed in shunt versus sham (Supplementary material online, *Figure S2D*). Neither the autophagy nor the ubiquitinated proteins exhibited any significant difference between chronic VO and sham controls (see the Supplementary material online, *Figure S3*).

Signalling pathways in volume overload

Classical markers of the fetal gene expression program were also differentially expressed in chronic VO vs. sham. Atrial natriuretic peptide (*Nppa*) expression was upregulated by 10-fold, whereas

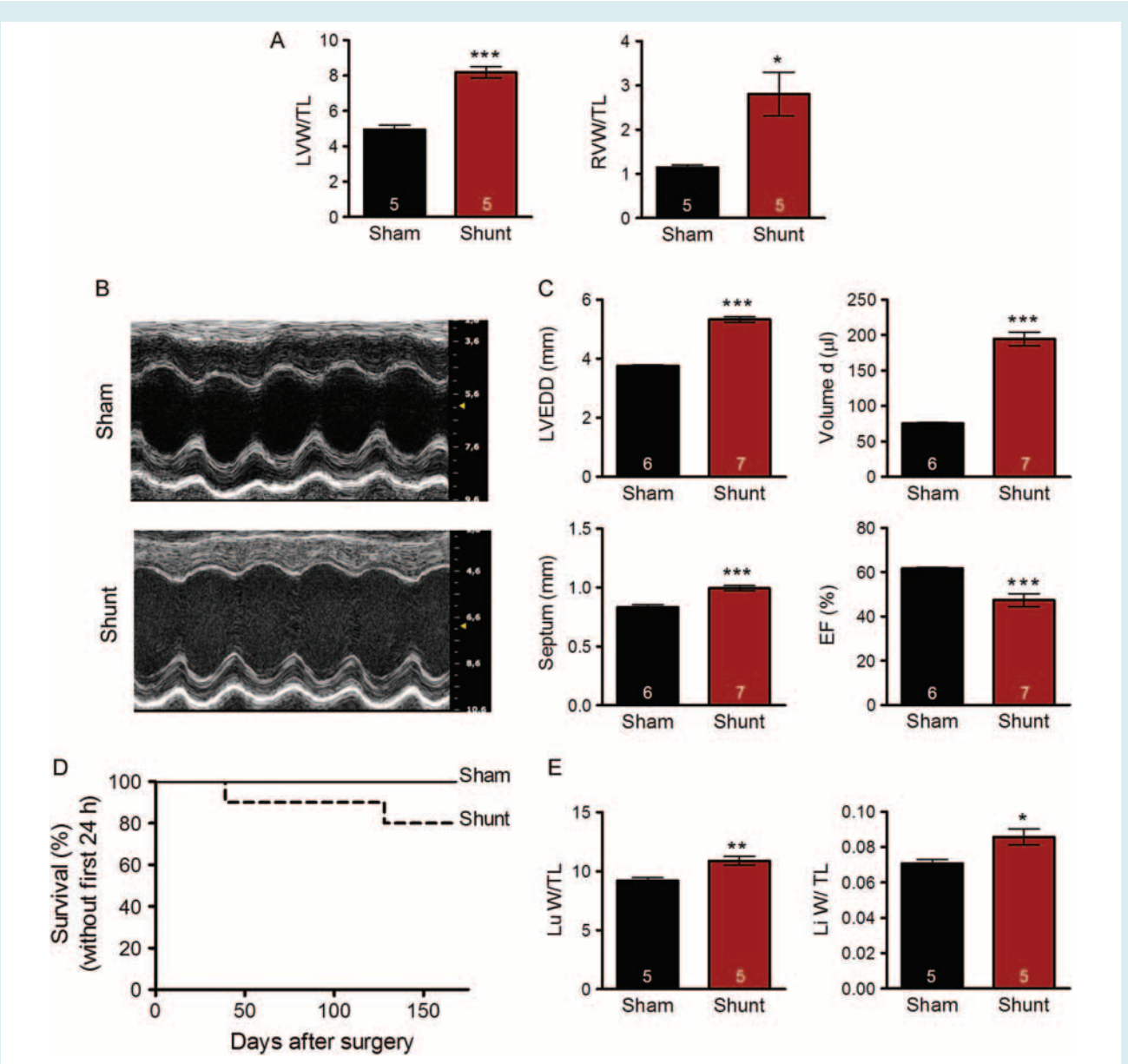

Figure 1 Cardiac dilatation, dysfunction, increased mortality and circulatory congestion in response to 20 weeks of volume overload. (A) Left ventricle weight-to-tibia length (LVW/TL) and right ventricle weight-to-tibia length (RVW/TL) ratios. (B) Echocardiographic M-mode images. (C) Average values for left ventricular end-diastolic diameter (LVEDD), left ventricular volume at diastole, septum thickness and ejection fraction (EF). (D) Kaplan–Meier curves depicting survival in sham- and shunt-operated mice ($n = 10$ mice per group). (E) Mean values for lung weight-to-tibia length (LuW/TL) and liver weight-to-tibia length (LiW/TL) ratios. Data are means ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus sham. Numbers in bars are number of mice.

brain natriuretic peptide (*Nppb*) showed an increase by 2.3-fold, along with a decrease in sarcoplasmic reticulum Ca^{2+} ATPase (*Serca2a*) expression to ≈80% of that in sham (*Figure 3A*). We observed a significant linear correlation between hypertrophy markers and structural/functional echocardiographic data (see the Supplementary material online, *Figure S4A,B*). Furthermore, the gene expression profiles of *Nppb*, *Nppa*, and *Serca2a* significantly correlated with one another (see the Supplementary material online, *Figure S4C*).

We analysed the classical HF signalling pathways by phospho-specific antibodies. We found that Akt showed a lower degree of phosphorylation in chronic VO compared with sham hearts (*Figure 3B*). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) as a negative regulator of Akt was unaltered in chronic VO, indicating a PTEN-independent mechanism of Akt inhibition. Interestingly, activation of the calcineurin/nuclear factor of activated T cells (NFAT) pathway, measured by *Rcan1.4* expression, and phosphorylation of CaMKIIδc were significantly

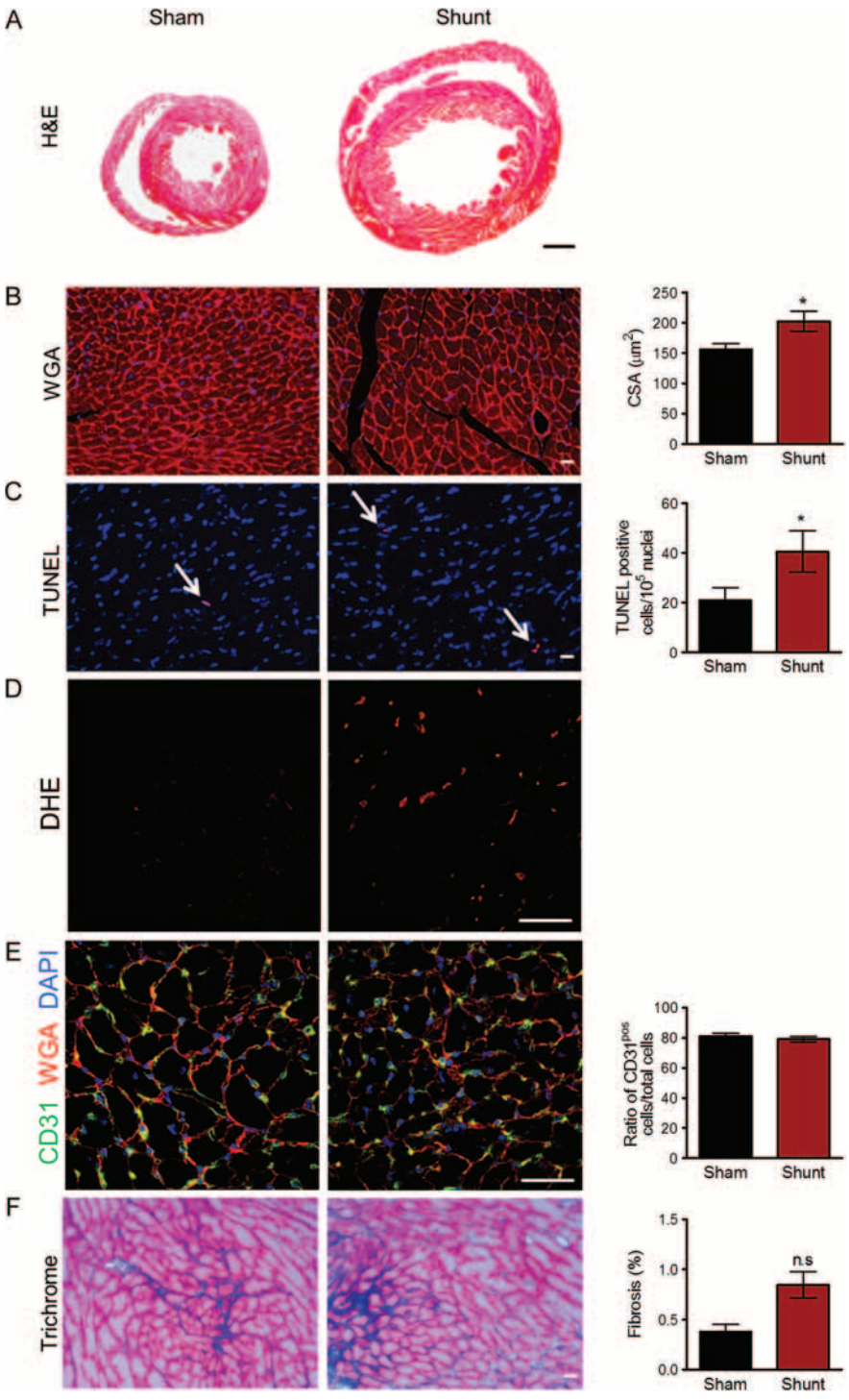

Figure 2 Pathological cardiac remodelling in long-term volume overload. (A) Haematoxylins–eosin (H&E)-stained transverse sections. Scale bar: 1 mm. (B) Representative photomicrographs illustrating ventricular myocyte cross-sections stained with wheat germ agglutinin (WGA) (left panel). Scale bar: 50 μm. Cross sectional area (CSA) quantification is shown in the right panel. (C) Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-assay (left panel), arrows indicate apoptotic nuclei. Bar: 50 μm. Quantification is shown in the right panel. (D) Reactive oxygen species production assessed by dihydroethidium (DHE) conversion to red fluorescent ethidium. Bar: 30 μm. (E) Capillary density expressed as the number of capillaries/total cells (left panel). Bar: 30 μm. Quantification of results is shown in the right panel. (F) Masson's trichrome staining (left panel). Bar: 50 μm. Quantification of results is shown in the right panel. Data are means ± SEM; $^{*}P < 0.05$ vs. sham. At least 4 mice/ group were analyzed.

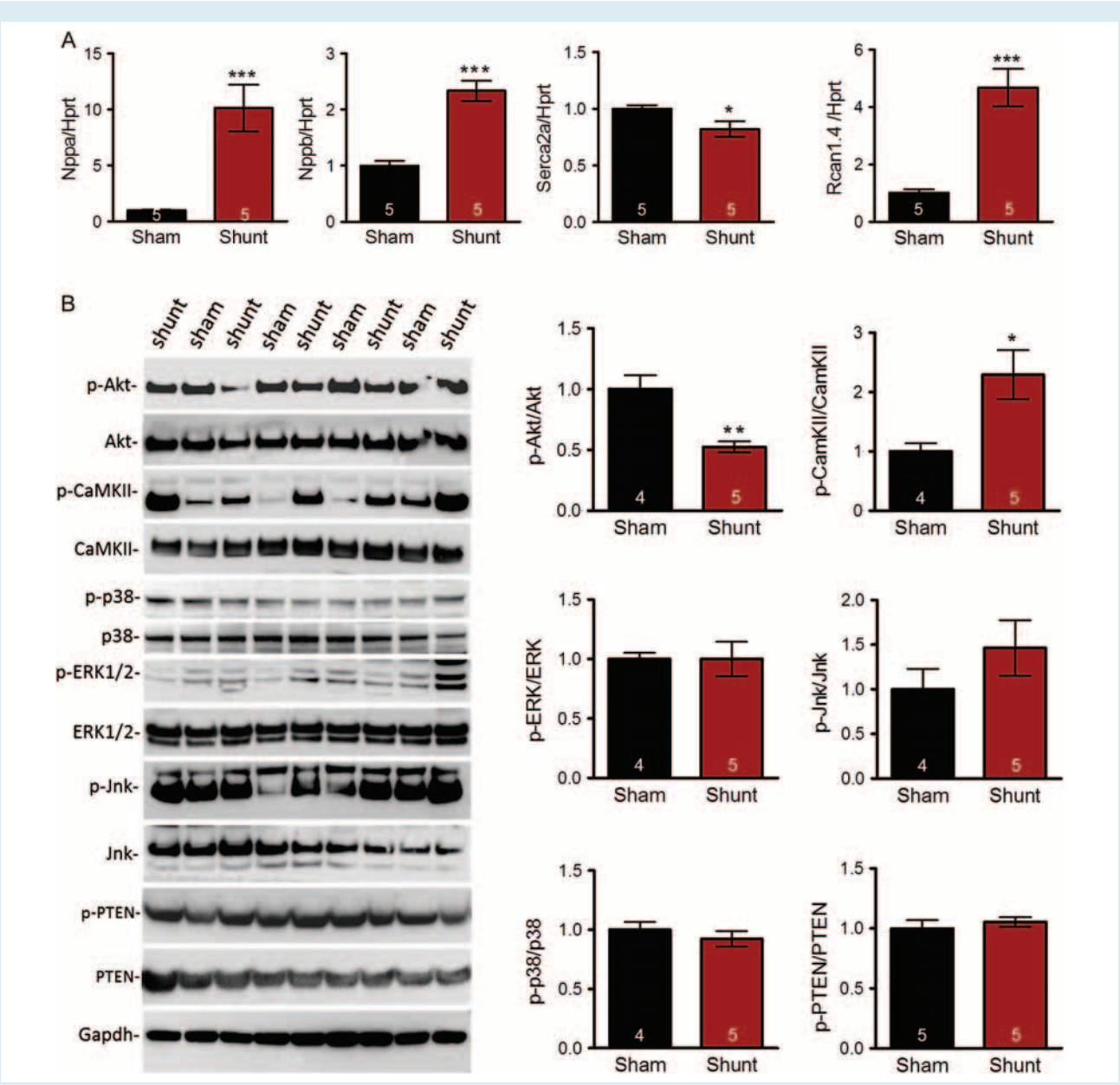

Figure 3 Effect of haemodynamic volume overload on classical hypertrophy signalling pathways. (A) Re-expression of fetal genes and expression of *Rcan1.4* in chronic volume overload. (B) Total protein and phosphorylation levels of classical signalling pathways mediating the development of heart failure. Original immunoblots (left panel), and phosphorylated protein levels normalized to the respective total proteins (right panel). Data are means ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. sham. Numbers in bars are number of mice; analysis per heart in duplicate.

increased in chronic VO (*Figure 3A,B*). The mitogen-activated protein kinases Jnk, P38 and extracellular-signal-regulated kinases 1/2 showed phosphorylation levels in VO comparable to those in sham hearts (*Figure 3B*).

Titin hypophosphorylation in chronic volume overload

Volume overload-induced myocardial stretch affects the elastic components of the cardiomyocytes, including titin's I-band region.[1] Titin-based myocardial stiffness is mainly determined by the relative expression levels of the cardiac titin isoforms N2B and N2BA,[2] and by phosphorylation of the elastic titin elements, N2-B unique sequence (N2-Bus) and PEVK-region (PEVK, proline, glutamate, valine, and lysine residues).[1] Therefore, we quantified titin isoform expression and phosphorylation in acute and chronic VO. Titin isoform composition remained unchanged in chronic shunt vs. sham hearts (see the Supplementary material online, *Figure S5B,C*). In contrast, all-titin phosphorylation was significantly

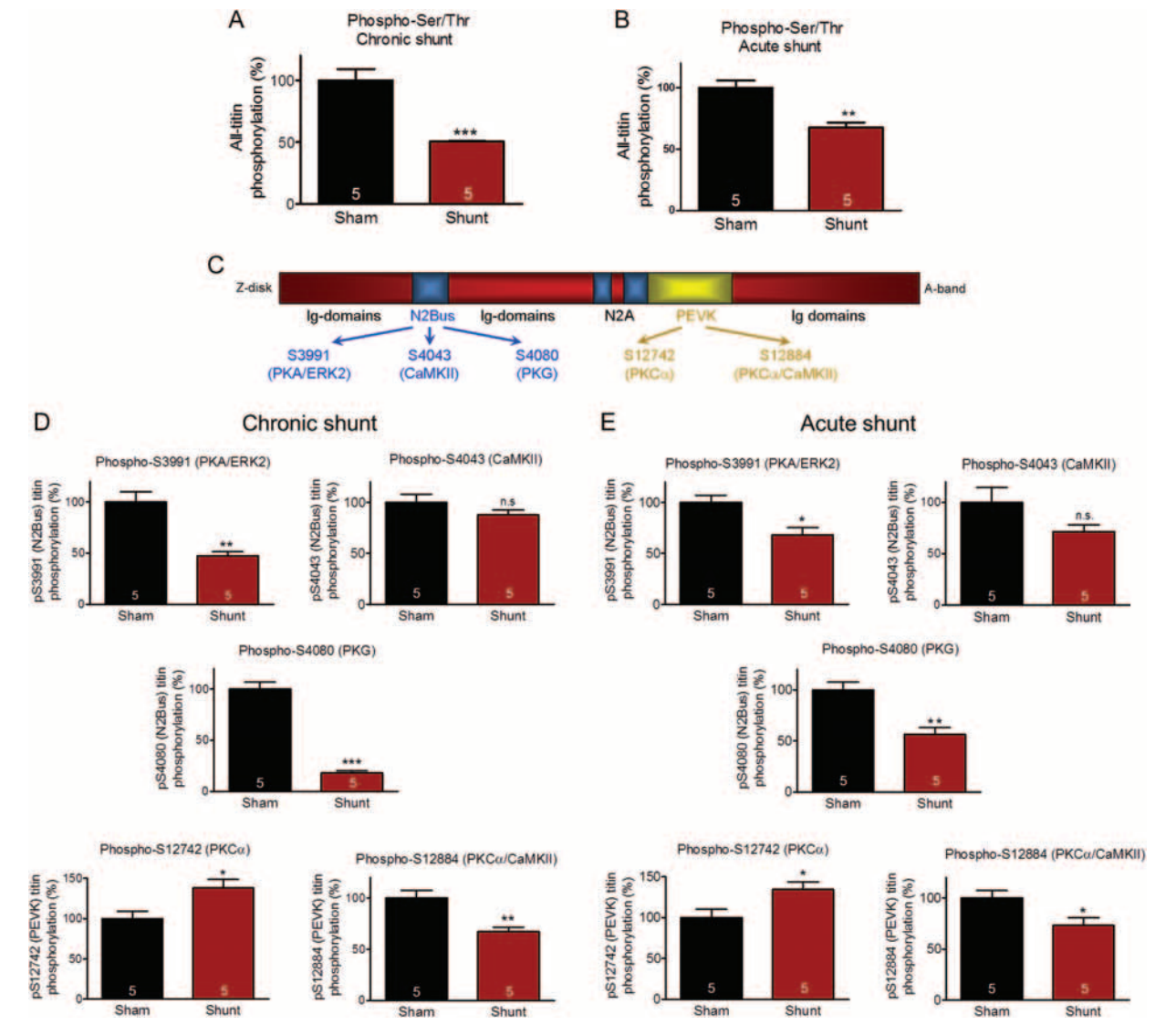

Figure 4 Cardiac titin phosphorylation in volume overload-subjected mice. (A,B) Graphs showing all-titin phosphorylation in chronic (A) and acute (B) shunt normalized to sham. (C) Schematic of extensible I-band titin region illustrating the epitope positions of the phosphospecific titin antibodies (mouse titin; UniProtKB #A2ASS6). (D,E) Graphs illustrating site-specific phosphorylation of titin N2B-unique sequence (N2Bus) and PEVK in chronic (D) and acute (E) shunt normalized to sham. Data are means ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. sham. Numbers in bars are number of mouse hearts; analysis per heart in duplicate.

reduced in chronic VO by ≈ 50% (*Figure 4A* and *Figure S5A*). Using affinity-purified phosphospecific antibodies, we assessed by western blot the phosphorylation status at conserved serines within the N2Bus (S3991, S4043, and S4080) and PEVK segments (S12742 and S12884) (*Figure 4C*). Compared with sham, chronic shunt hearts exhibited significant hypophosphorylation at the S3991, S4080, and S12884 sites, hyperphosphorylation at the S12742 site, but similar phosphorylation at the S4043 site (*Figure 4D* and *Figure S5A*). These changes suggest a stiffer titin.

In the early phase of experimental VO at 1 week after shunt, we observed a similar pattern of altered total and site-specific titin phosphorylation, albeit to a lesser extent. All-titin phosphorylation was 100 ± 5.85 in sham and 67.61 ± 4.19 in shunt ($P < 0.01$) (*Figure 4B,E*).

Akt is involved in the early adaptation to volume overload

Because Akt is activated in the early phase of experimental VO,[4] we tested the hypothesis that Akt is involved in the early adaptive response to VO and could protect against HF development. Comparison of *Akt*$^{-/-}$ mice with their wild-type (WT) littermates showed a higher mortality after VO in *Akt*$^{-/-}$ mice (*Figure 5A*). At 4 weeks after shunt, the degree of cardiac hypertrophy, measured

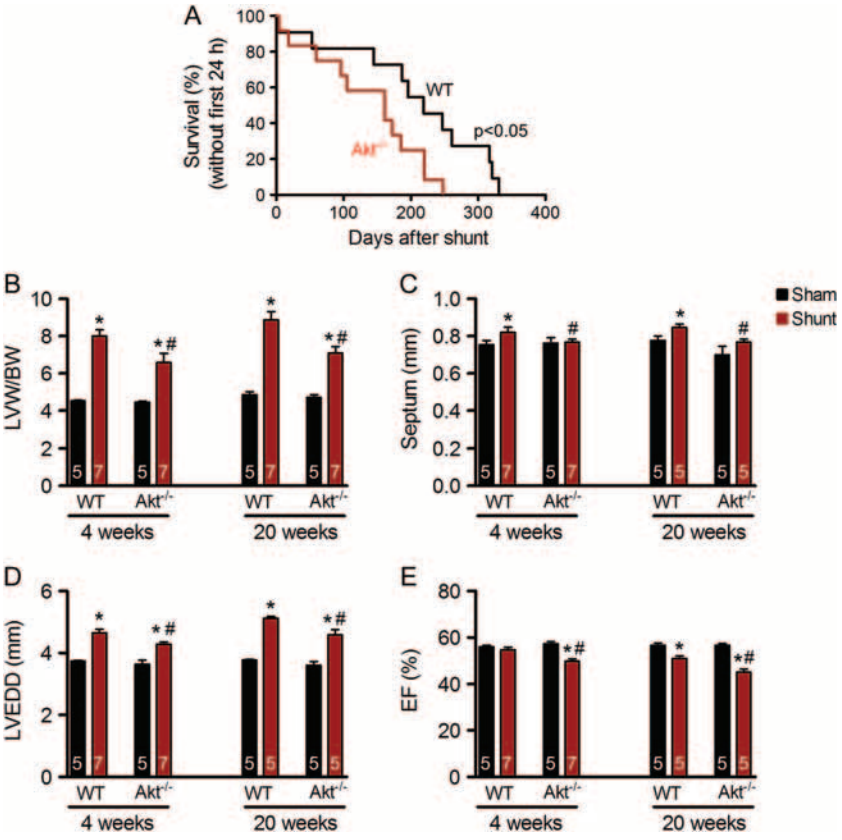

Figure 5 $Akt^{-/-}$ mice exhibit an increased mortality and greater cardiac decompensation upon volume overload than wild-type (WT) mice. (A) Kaplan–Meier plots showing survival rates in the indicated genotypes ($n = 11–12$ mice per group). (B–E) Left ventricular weight-to-body weight (LVW/BW) (B), septum thickness (C), left ventricular end-diastolic diameter (LVEDD) (D), and ejection fraction (EF) (E) in WT and $Akt^{-/-}$ mice at 4 weeks and 20 weeks of volume overload. Data are means ± SEM; $^{*}P < 0.05$ vs. corresponding sham, $^{\#}P < 0.05$ vs. WT shunt. Numbers in bars are number of mice.

by either left ventricular weight-to-body weight ratio or by septum width, was reduced in $Akt^{-/-}$ vs. WT animals (*Figure 5B,C* and Supplementary material online, *Table S2*). In addition, LV dilatation was reduced compared with control, indicating a misbalanced adaptation of $Akt^{-/-}$ mice to VO (*Figure 5D* and Supplementary material online, *Table S2*). The cardiac function measured by EF was slightly reduced in $Akt^{-/-}$ mice by this time-point (*Figure 5E*; Supplementary material online, *Table S2*). After 20 weeks of VO, impaired cardiac function was seen in WT shunt mice, but this was more pronounced in $Akt^{-/-}$ shunt animals, indicating a loss of protective remodelling in $Akt^{-/-}$ mice (*Figure 5E* and Supplementary material online, *Table S3*).

Discussion

Volume overload by aortocaval shunt was reported to exhibit an early adaptive phenotype with late HF development.[4] In the present study, we showed that transition to HF in experimental VO (i) is associated with decreased Akt, increased activation of CaMKII and calcineurin, and apoptosis induction, (ii) is accelerated by Akt deletion, and (iii) is associated with titin hypophosphorylation and lack of interstitial fibrosis suggesting increased sarcomeric vs. interstitial stiffness. Thus, transition to failure in VO may result from myocyte loss and myocyte dysfunction caused by increased sarcomeric stiffness.

Myocardial fibrosis

In contrast to other forms of HF (PO, myocardial infarction, dilated cardiomyopathy), VO did not induce fibrosis. Previous analyses in rat and dog models showed that there is relatively little change in collagen synthesis, but an abnormal production of non-collagen extracellular matrix.[12] This seems to be also true in humans.[13] In the early phase of VO, a controlled dilatation of the heart is required for a successful adaptation. Dilatation is closely regulated by matrix metalloproteinases and tissue inhibitor of metalloproteinases that are partly secreted by inflammatory cells, especially mast cells.[14] It was suggested that during the progression from the compensated state to HF these processes are more and more dysregulated, so that further dilatation and, over time, impairment of cardiac function occurs.[15]

Oxidative stress

It has been reported that oxidative stress plays a role in the VO model.[16] Here, we observed a robust generation of superoxide and thus, increased oxidative stress by DHE staining in the myocardium of the VO model compared with controls. Increased superoxide concentration reduces the bioavailability of NO by chemical inactivation to form toxic peroxynitrite. Markedly increased protein nitration was observed through nitrotyrosine staining. Increased oxidative stress activates a broad variety of hypertrophy signalling kinases and transcription factors and induces apoptosis, resulting in a potentially deleterious effect on myocardial contractility.[17] Oxidative stress may also be linked to extracellular matrix (ECM) remodelling, as loss of interstitial collagen seems to induce oxidative stress.[18] In contrast, treatment of VO animals with the antioxidant mitoubiquinone (MitoQ) did not prevent LV dilatation and HF.[16] Therefore, it is unclear whether oxidative stress is the result of dilatation or is also a player in the development of HF. Further studies are needed to resolve this issue.

Apoptosis

The heart is very sensitive to myocyte death, and very low levels of myocyte apoptosis were sufficient to cause pathological ventricular remodeling.[19] We showed that transition to HF in VO is associated with increased apoptotic cell death. Furthermore, we demonstrated decreased phosphorylation of Akt, increased CaMKIIδ and elevated oxidative stress in remodelled hearts exposed to VO. These alterations may trigger downstream mechanisms that increase myocardial apoptosis after shunt.

Titin hypophosphorylation

Titin is stretched by increased preload as it occurs in our VO model. Titin stiffness has recently been reported to be increased after 4 weeks of VO.[20] This could lead to diastolic dysfunction and progression of HF or, alternatively, this could have a protective effect by limiting ventricular dilatation. The authors showed the presence of dilatation in a mouse model expressing very long, compliant titin, compared with WT titin mouse hearts; however, normal cardiac function was maintained.[20] Markers of survival and other markers of HF were not analysed in this study, leaving questions unanswered about the role of titin stiffness in maladaptive vs. protective cardiac remodelling in VO.

Titin stiffness can substantially be modified by altered isoform expression and phosphorylation. Patients with end-stage HF caused by dilated cardiomyopathy were shown to have decreased or unaltered total titin phosphorylation.[21,22] Site-specific phosphorylation at titin spring elements showed a consistent pattern in dilated cardiomyopathy and hypertrophic cardiomyopathy patients, as well as in dog hearts with early diastolic HF: N2Bus phosphorylation was decreased and PEVK phosphorylation (partially) increased.[22,23] Both these alterations are predicted to cause increased titin stiffness.[21,24,25] The same pattern of changes in titin phosphorylation could be detected in our chronic VO model. Interestingly, the alterations were already present after 1 week of VO. This finding is consistent with a role of titin stiffness in limiting cardiac dilatation in VO, particularly in the absence of fibrosis. This would be in contrast to PO-induced HF, where global stiffness of the heart is increased because of fibrosis while titin stiffness is reduced, perhaps as a compensatory mechanism. However, increased sarcomeric stiffness in VO may also cause contractile dysfunction at the level of the contractile machinery. Interestingly, the phosphorylation of the PKA (S3991) and PKG (S4080) sites of titin was decreased in our VO model, suggesting that the local activities of PKA and/or PKG might be reduced in the shunt model. The phosphorylation of the two CaMKIIδc sites on titin was unaltered (S4043) or even decreased (S12884) in our model, whereas phosphorylation of both these sites was found elsewhere to be increased in human failing hearts.[26] These observations suggest a limited role of CaMKIIδc in VO-induced titin phosphorylation. Taken together, our findings point to a scenario whereby increased titin stiffness via hypophosphorylation limits cardiac dilatation in VO. Whether increased titin stiffness contributes to HF by causing diastolic or systolic dysfunction of the contractile machinery needs to be analysed in further studies.

Akt is critically involved in the early adaptation to volume overload

Previously, we showed that Akt is activated at an early time-point of experimental VO, whereas other pro-hypertrophic pathways such as calcineurin, CaMKII, or mitogen-activated protein kinase (MAPK) do not seem to play a role.[4] Here, we analysed the role of Akt activation in VO hypertrophy and the development of HF. The *Akt*$^{-/-}$ mice showed a decreased cardiac hypertrophic response with early HF development and a higher mortality rate compared with WT mice. Thus, VO may have similar effects on cardiac hypertrophy as exercise, which was shown to have a lesser impact on the (physiological) development of hypertrophy in *Akt*$^{-/-}$ than in WT mice.[7,27] Pressure overload induced an increase in heart mass in *Akt*$^{-/-}$ mice.[7] This suggests a beneficial role for Akt activation in cardiac remodelling, both by limiting maladaptive as well as mediating adaptive cardiac hypertrophy under different stress conditions. In our VO model, early Akt activation contributes to beneficial hypertrophy whereas reduced activation, indicated by a decreased phosphorylation status at a later time-point, seems to contribute to the progression of HF. The changes in Akt signalling seem to be independent on PTEN. This indicates that upstream mechanisms of integrin and focal adhesion kinase (FAK) may play a more prominent role in early activation and possibly also in late inhibition of Akt.

Signalling pathways in chronic volume overload

In chronic VO, we demonstrated re-expression of the fetal gene program (upregulation of *Nppa* and *Nppb*). Interestingly, the upregulation of *Nppa* was much more pronounced compared with that of *Nppb*, while in the previously published PO model, *Nppb* was upregulated more than *Nppa*. This finding indicates that at least part of the deregulation in chronic VO is different from that in PO.

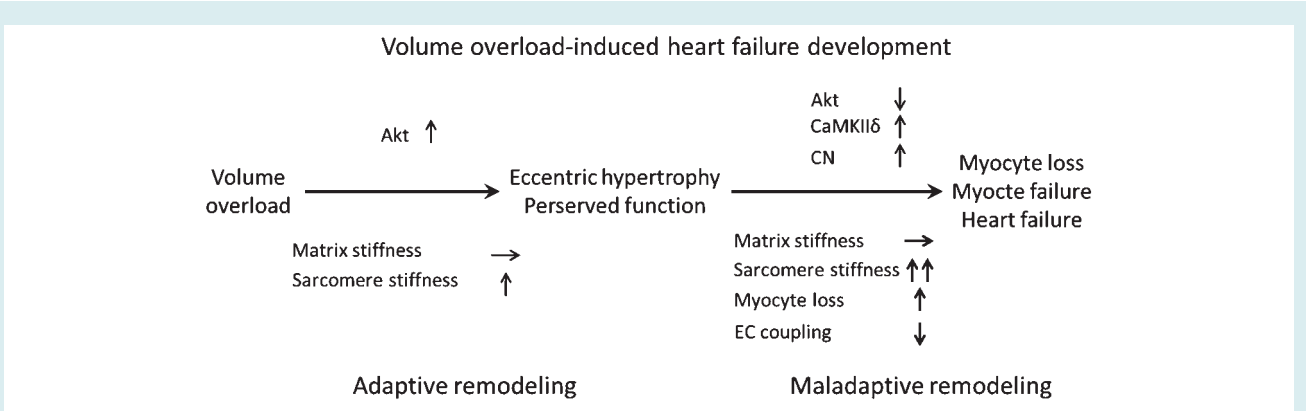

Figure 6 Schematic diagram for the transition to heart failure in volume overload. Akt, protein kinase B; CaMKIIδ, calcium/calmodulin-dependent protein kinase IIδ; CN, calcineurin; EC coupling, excitation–contraction coupling. Evidence is provided in this and our previous publication.[4] Preserved matrix stiffness owing to unchanged fibrosis; increased sarcomere stiffness owing to titin hypophosphorylation; increased apoptosis indicated by increased Bax/Bcl-2 and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL); deteriorated EC-coupling indicated by decreased Serca2a and increased CaMKIIδ (CaMKIIδ modifies EC-coupling).[29]

Analyses of the classical HF signalling pathways in chronic VO showed that calcineurin and CaMKIIδc are activated. As shown previously, CaMKII-knockout rescued PO-subjected mice from apoptosis and HF.[28] Therefore, in addition to decreased Akt activity, increased CaMKII activity may be the key alteration in signalling to induce failure in VO. We conclude that deactivation of Akt and activation of CaMKII may induce the transition to HF. In PO these changes seem to occur immediately after load elevation.[4] In contrast, this kinase switch takes place later in VO and thus is not directly induced by elevated preload. Moreover, the signal switch is associated with increased oxidative stress and apoptosis, which may be important triggers for the transition to HF in VO. Unlike PO-induced HF, VO-induced HF is not associated with increased fibrosis. Lack of fibrosis may be part of the adaptive mechanism of increased LV volume. One may speculate that lack of matrix stiffness is compensated by increased sarcomeric stiffness owing to titin hypophosphorylation. While this may be compensatory at the level of myocardial tissue stability, it may be detrimental regarding contractile function at the sarcomeric level (*Figure 6*).[29]

The fact that early development of HF in experimental PO[4] and late HF progression in induced VO are associated with deactivation of Akt and activation of CaMKII supports the notion that mechanisms which activate Akt and/or suppress CaMKII may be important therapeutic targets.

Supplementary Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Volume overload induces maladaptive remodelling. Serial echo showing increased left ventricular end diastolic diameter (LVEDD) (A) and decreased ejection fraction (EF) (B) upon shunt-induced volume overload.

Figure S2. (A) Attenuated anti-apoptotic signalling in chronic volume overload. (B) Increased oxidative stress in chronic shunt.

Figure S3. Normal level of autophagy and ubiquitinated proteins in chronic volume overload in comparison to sham hearts. (A,B) Western blots for ubiquitinated proteins.

Figure S4. Correlation coefficient of the expressions of the hypertrophy-related markers to the echocardiographic left ventricular end diastolic diameter (LVEDD) (A) and ejection fraction (EF) data (B) and to each other (C).

Figure S5. Titin hypophosphorylation but unchanged titin isoform composition. (A) Representative titin gel bands. (B,C) Average titin-isoform composition in chronic (B) and acute (C) shunt hearts relative to controls.

Method S1. Supplementary materials and methods.

Table S1. Echocardiographic measurements of sham- and shunt-operated mice at 20 weeks after aortocaval shunt.

Table S2. Echocardiographic parameters of wild-type and Akt$^{-/-}$ mice at 4 weeks after shunt

Table S3. Echocardiographic parameters of wild-type and Akt$^{-/-}$ mice at 20 weeks after shunt.

Acknowledgments

We gratefully acknowledge the technical assistance of Sabrina Koszewa and Marion von Frieling-Salewsky.

Funding

This work was supported the German Research Foundation (SFB 1002 to K.T., W.A.L., and G.H.). M.S. is funded by the German Research Foundation DFG grant no. IRTG1816.

Conflict of interest: none declared.

References

1. Linke WA, Hamdani N. Gigantic business: titin properties and function through thick and thin. *Circ Res* 2014;**114**:1052–1068.
2. Neagoe C, Kulke M, del Monte F, Gwathmey JK, de Tombe PP, Hajjar RJ, Linke WA. Titin isoform switch in ischemic human heart disease. *Circulation* 2002;**106**:1333–1341.

3. von Lewinski D, Stumme B, Fialka F, Luers C, Pieske B. Functional relevance of the stretch-dependent slow force response in failing human myocardium. *Circ Res* 2004;**94**:1392–1398.
4. Toischer K, Rokita AG, Unsöld B, Zhu W, Kararigas G, Sossalla S, Reuter SP, Becker A, Teucher N, Seidler T, Grebe C, Preuss L, Gupta SN, Schmidt K,Lehnart SE, Krüger M, Linke WA, Backs J, Regitz-Zagrosek V, Schäfer K, Field LJ, Maier LS, Hasenfuss G. Differential cardiac remodeling in preload versus afterload. *Circulation* 2010;**122**:993–1003.
5. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007;**129**:1261–1274.
6. Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA, Gu Y, Dalton N, Chung C, Latronico MV, Napoli C, Sadoshima J, Croce CM, Ross J Jr. Akt induces enhanced myocardial contractility and cell size *in vivo* in transgenic mice. *Proc Natl Acad Sci U S A* 2002;**99**:12333–12338.
7. DeBosch B, Treskov I, Lupu TS, Weinheimer C, Kovacs A, Courtois M, Muslin AJ. Akt1 is required for physiological cardiac growth. *Circulation* 2006;**13**: 2097–2104.
8. Haq S, Choukroun G, Lim H, Tymitz KM, del Monte F, Gwathmey J, Grazette L, Michael A, Hajjar R, Force T, Molkentin JD. Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. *Circulation* 2001;**103**:670–677.
9. Matsui T, Li L, Wu JC, Cook SA, Nagoshi T, Picard MH, Liao R, Rosenzweig A. Phenotypic spectrum caused by transgenic overexpression of activated Akt in the heart. *J Biol Chem* 2002;**277**:22896–22901.
10. Taniyama Y, Ito M, Sato K, Kuester C, Veit K, Tremp G, Liao R, Colucci WS, Ivashchenko Y, Walsh K, Shiojima I. Akt3 overexpression in the heart results in progression from adaptive to maladaptive hypertrophy. *J Mol Cell Cardiol* 2005;**38**:375–385.
11. Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R, Colucci WS, Walsh K. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest* 2005;**115**:2108–2118.
12. Borer JS, Truter S, Herrold EM, Falcone DJ, Pena M, Carter JN, Dumlao TF, Lee JA, Supino PG. Myocardial fibrosis in chronic aortic regurgitation: molecular and cellular responses to volume overload. *Circulation* 2002;**105**:1837–1842.
13. Piper C, Schultheiss HP, Akdemir D, Rudolf J, Horstkotte D, Pauschinger M. Remodeling of the cardiac extracellular matrix differs between volume- and pressure-overloaded ventricles and is specific for each heart valve lesion. *J Heart Valve Dis* 2003;**12**:592–600.
14. Ryan TD, Rothstein EC, Aban I, Tallaj JA, Husain A, Lucchesi PA, Dell'Italia LJ. Left ventricular eccentric remodeling and matrix loss are mediated by bradykinin and precede cardiomyocyte elongation in rats with volume overload. *J Am Coll Cardiol* 2007;**49**:811–821.
15. Zheng J, Chen Y, Pat B, Dell'italia LA, Tillson M, Dillon AR, Powell PC, Shi K, Shah N, Denney T, Husain A, Dell'Italia LJ. Microarray identifies extensive downregulation of noncollagen extracellular matrix and profibrotic growth factor genes in chronic isolated mitral regurgitation in the dog. *Circulation* 2009; **119**:2086–2095.
16. Yancey DM, Guichard JL, Ahmed MI, Zhou L, Murphy MP, Johnson MS, Benavides GA, Collawn J, Darley-Usmar V, Dell'Italia LJ. Cardiomyocyte mitochondrial oxidative stress and cytoskeletal breakdown in the heart with a primary volume overload. *Am J Physiol Heart Circ Physiol* 2015;**308**:H651–H663.
17. Sabri A, Hughie HH, Lucchesi PA. Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. *Antioxid Redox Signal* 2003;**5**:731–740.
18. Ulasova E, Gladden JD, Chen Y, Zheng J, Pat B, Bradley W, Powell P, Zmijewski JW, Zelickson BR, Ballinger SW, Darley-Usmar V, Dell'italia LJ. Loss of interstitial collagen causes structural and functional alterations of cardiomyocyte subsarcolemmal mitochondria in acute volume overload. *J Mol Cell Cardiol* 2011;**50**:147–156.
19. Wencker D, Chandra M, Nguyen K, Miao W, Garantziotis S, Factor SM, Shirani J, Armstrong RC, Kitsis RN. A mechanistic role for cardiac myocyte apoptosis in heart failure. *J Clin Invest* 2003;**111**:1497–1504.
20. Hutchinson KR, Saripalli C, Chung CS, Granzier H. Increased myocardial stiffness due to cardiac titin isoform switching in a mouse model of volume overload limits eccentric remodeling. *J Mol Cell Cardiol* 2015;**79**:104–114.
21. Krüger M, Kötter S, Grützner A, Lang P, Andresen C, Redfield MM, Butt E, dos Remedios CG, Linke WA. Protein kinase G modulates human myocardial passive stiffness by phosphorylation of the titin springs. *Circ Res* 2009;**104**: 87–94.
22. Kötter S, Gout L, Von Frieling-Salewsky M, Müller AE, Helling S, Marcus K, Dos Remedios C, Linke WA, Krüger M. Differential changes in titin domain phosphorylation increase myofilament stiffness in failing human hearts. *Cardiovasc Res* 2013;**99**:648–656.
23. Hamdani N, Bishu KG, von Frieling-Salewsky M, Redfield MM, Linke WA. Deranged myofilament phosphorylation and function in experimental heart failure with preserved ejection fraction. *Cardiovasc Res* 2013;**97**:464–471.
24. Krüger M, Linke WA. Protein kinase-A phosphorylates titin in human heart muscle and reduces myofibrillar passive tension. *J Muscle Res Cell Motil* 2006;**27**:435–444.
25. Hidalgo C, Hudson B, Bogomolovas J, Zhu Y, Anderson B, Greaser M, Labeit S, Granzier H. PKC phosphorylation of titin's PEVK element: a novel and conserved pathway for modulating myocardial stiffness. *Circ Res* 2009;**105**:631–638.
26. Hamdani N, Krysiak J, Kreusser MM, Neef S, Dos Remedios CG, Maier LS, Krüger M, Backs J, Linke WA. Crucial role for Ca2(+)/calmodulin-dependent protein kinase-II in regulating diastolic stress of normal and failing hearts via titin phosphorylation. *Circ Res* 2013;**112**:664–674.
27. Walsh K. Akt signaling and growth of the heart. *Circulation* 2006;**113**:2032–2034.
28. Ling H, Zhang T, Pereira L, Means CK, Cheng H, Gu Y, Dalton ND, Peterson KL, Chen J, Bers D, Brown JH. Requirement for Ca^{2+}/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiachypertrophy to heart failure in mice. *J Clin Invest* 2009;**119**:1230–1240.
29. Dzhura I, Wu Y, Colbran RJ, Balser JR, Anderson ME. Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels. *Nat Cell Biol* 2000;**2**:173–177.