Differential Activation of Signal Transduction Pathways in Human Hearts With Hypertrophy Versus Advanced Heart Failure

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Background—Left ventricular failure is commonly preceded by a period of hypertrophy. Intriguingly, many of the signaling pathways that have been implicated in the regulation of hypertrophy, including the 3 mitogen-activated protein kinases (MAPKs: extracellular signal-regulated kinase, stress-activated protein kinase, and p38), protein phosphatase, calcineurin, and the protein kinase Akt and its target glycogen synthase kinase-3 (GSK-3), also regulate the apoptotic response.

Methods and Results—To understand the mechanisms that might regulate the progression of heart failure, we analyzed the activity of these signaling pathways in the hearts of patients with advanced heart failure, patients with compensated cardiac hypertrophy, and normal subjects. In patients with hypertrophy, neither the MAPK nor the Akt/GSK-3 pathways were activated, and the dominant signaling pathway was calcineurin. In failing hearts, calcineurin activity was increased but less so than in the hypertrophied hearts, and all 3 MAPKs and Akt were activated (and, accordingly, GSK-3β was inhibited), irrespective of whether the underlying diagnosis was ischemic or idiopathic cardiomyopathy.

Conclusions—In the failing heart, there is a clear prohypertrophic activity profile, likely occurring in response to increased systolic wall stress and neurohormonal mediators. However, with the activation of these hypertrophic pathways, potent proapoptotic and antiapoptotic signals may also be generated. Therapies directed at altering the balance of activity of these signaling pathways could potentially alter the progression of heart failure. (Circulation. 2001;103:670-677.)

Key Words: calcineurin ■ cardiomyopathy ■ mitogen-activated protein kinases

In response to pathological stimuli, the myocardium can undergo adaptive hypertrophic growth to augment cardiac function. Although this response is initially beneficial, sustained cardiac hypertrophy is a leading predictor for the development of heart failure. In recent years, intensive investigation has centered around characterizing intracellular regulatory pathways that are associated with hypertrophy and heart failure in an attempt to design novel pharmacological strategies that may alleviate these responses.

Several intracellular signaling pathways have been implicated in the induction of cardiac hypertrophy. These include the small GTP binding proteins Ras and Rac, the Gq subunit of heterotrimeric G proteins, the 3 main branches of the mitogen-activated protein kinase (MAPK) signaling cascades (extracellular signal-regulated kinases [ERKs], stress-activated protein kinases [SAPKs], and p38s), protein kinase C isoforms, and calcineurin (Cn). Although these pathways/factors may play important regulatory roles in the development of cardiac hypertrophy in experimental animals, virtually nothing is known about their activation state or regulatory roles in stable, compensated hypertrophy in humans, and little is known about their roles in the failing human heart.

Several reports have examined the associations between intracellular signaling pathways and human heart failure. One showed an association between various protein kinase C isoforms and heart failure. More recently, 2 of the MAPK pathways, SAPK and p38 (but not ERK), were reported to be activated in human heart failure due to coronary artery disease (CAD). Finally, studies on Cn protein levels in failing human hearts have produced disparate results, and none have examined Cn activity. Most importantly, all of these reports have focused on individual pathways and, although valuable information has been gained, this approach does not allow for the development of a sense of the relative importance of the
various pathways in the progression of disease or their roles at different points in the disease.

Recent evidence suggests that apoptosis may also play an important role in the transition from hypertrophy to heart failure and the progression of failure. The serine/threonine kinase protein kinase B (PKB)/Akt pathway transduces anti-apoptotic “survival” signals in a number of cells, including cardiomyocytes. Thus, it is essential to understand the activation state of this kinase to better understand the mechanisms underlying cardiomyocyte apoptosis in the failing heart, but Akt activity in failing (or hypertrophied) human hearts has not been studied.

In the present study, we examined the activity of the following 8 signaling molecules in the hearts of patients with compensated hypertrophy or with advanced heart failure: Cn, SAPK, p38, ERK, Akt, 2 activators of Akt (the insulin-like growth factor-1 [IGF-1] and ErbB2 receptors), and the Akt target glyycogen synthase kinase-3 (GSK-3). All of these molecules have been implicated in both hypertrophic and either proapoptotic or antiapoptotic responses. Thus, it is essential to understand the activity profiles of altered regulation in stable hypertrophy versus failure failure suggest that different molecules could serve as targets for therapies directed at specific phases of the disease.

### Methods

#### Western Blotting Procedure

Lysates were matched for protein, separated by SDS-PAGE, and transferred to nitrocellulose membranes, which were probed with the antibodies described below. Antibody binding was detected with chemiluminescence.

The antibodies employed were as follows: anti–c-Jun NH$_2$-terminal kinase (JNK; recognizing all SAPK/JNK isoforms), anti-p38, anti-phosphotyrosine (PY20), and anti-ERK-1/-2 from Santa Cruz Biotechnology; anti-GSK-3β and anti-IGF-1 receptor α subunit (IGF-1Rα) from Transduction Laboratories; anti-PKB/Akt from New England Biolaboratories; and anti-ErbB2 from Neomarker.

The phospho-specific antibodies we used were as follows: dual phospho-specific ERK, dual phospho-specific p38, phospho Ser 473 PKB/Akt, and phospho-STAT3 from New England Biotechnology and dual phospho-specific SAPK/JNK from Promega.

To measure total CnA (catalytic subunit) protein content, 2 separate antibodies that recognize either the N-terminus (Chemicon) or C-terminus (Transduction Laboratories) were used. CnAα and Aβ isoform-specific antibodies were also employed (Santa Cruz). Western blots were processed using enhanced chemiluminescence (Amersham), and data were quantified with blue fluorescence imaging on a PhosphorImager. Cn signal intensity was normalized to GAPDH signal. Protein degradation was monitored with an antibody to protein kinase Cδ (Santa Cruz), and 3 samples, 2 failing and 1 normal, were excluded from analysis.

#### Immune Complex Kinase Assays

Immune complex kinase assays for ERK-1/-2, SAPK, p38, and GSK-3β activity were performed as described previously using the following substrates: GST-c-Jun(1–135) for SAPK, GST-ATF-2(8–94) for p38, myelin basic protein for ERK, and glycogen synthase peptide-2 (Upstate Biotechnology) for GSK-3β.

#### Cn Phosphatase Assay

Cn phosphatase assays were performed on human left ventricular free wall samples exactly as described previously.

#### Statistical Analysis

All data are presented as mean±SEM. Differences between values were evaluated for statistical significance using a non-paired Student’s $t$ test or 1-way ANOVA followed by Bonferroni’s multiple comparison test when appropriate. $P<0.05$ was considered statistically significant.

### Results

#### Patient Characteristics

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Cn Assay</th>
<th>Kinase Assay</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hypertrophy</td>
</tr>
<tr>
<td>Age, y</td>
<td>52±8.2</td>
<td>48±7.8</td>
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<tr>
<td>Male sex, %</td>
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<td>60</td>
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<td>Diagnosis, n</td>
<td>HTN</td>
<td>5</td>
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<tr>
<td></td>
<td>CAD</td>
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HTN indicates hypertension; CAD, coronary artery disease; and DCM, idiopathic dilated cardiomyopathy. Age is expressed as mean±SD.

The donor hearts that served as the controls (n=9) were taken from patients who were initially considered to be donors, with no history or findings of evidence of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease. The hypertrophied hearts (n=9) were taken from patients who were initially considered to be donors, with no history or findings of underlying cardiac disease.

The hearts explanted from patients with heart failure before cardiac transplantation (n=22), underlying diagnoses were CAD (n=11) and idiopathic dilated cardiomyopathy (n=11). The Table presents patient characteristics in the various groups. We had insufficient samples to perform all of the
assays in all of the hearts. Therefore, the Table presents the hypertrophied and failing hearts that were assayed for Cn activity and the hypertrophied and failing hearts that were assayed for kinase activity separately, with their respective control groups. The groups were well-matched for age and reasonably well-matched for sex.

Cn Activity in Hypertrophied and Failing Human Hearts

We first examined the activation status of Cn. There were significant increases in Cn enzymatic activity in both the hypertrophied (2.7-fold) and failing heart samples (1.7-fold) ($P=0.0005$ and $P=0.03$, respectively; Figure 1A), although activity was significantly greater in hypertrophied hearts compared with failing hearts ($P=0.03$; Figure 1A).

We next quantified Cn expression by Western blotting with 4 different CnA antibodies. Both of the pan-CnA antibodies and a CnA$\beta$-specific antibody demonstrated a subtle but significant increase in Cn protein in both hypertrophied and failing hearts (Figures 1B, 1C, and 1D). In contrast, CnA$\alpha$ was weakly detectable and was not changed in abundance (Figure 1C and 1D). CnA protein was elevated by $1.82\pm0.36$-fold ($P=0.05$) and $1.5\pm0.28$-fold ($P<0.05$) in hypertrophied and failing hearts, respectively. To determine CnA-specific activity, we normalized enzymatic activity to expression level

Figure 1. Cn activity and protein content in failing and hypertrophied human hearts. A, Cn phosphatase activity was measured in normal (control), hypertrophied, and failing hearts. There were significant increases in activity in hypertrophied and failing hearts. B, Quantification of CnA expression was determined by Western blotting with a pan-CnA antibody (Chemicon) and normalized to GAPDH expression. For these studies, lysates from an additional 4 control, 3 hypertrophied, and 3 failing hearts were subjected to Western blotting. C, Representative Western blot for Cn protein content in hypertrophied human heart samples. Extracts were Western-blotted against pan-CnA antibodies from 2 vendors (Chemicon [Chem] and Transduction Laboratories [TL]) or $\alpha$ and $\beta$ isoform-specific antibodies (Santa Cruz [SC]). GAPDH was run as a loading control. Brain extract was run to confirm Cn identity. D, Representative Western blot for Cn protein content in failing human heart samples. Same antibodies as those in C were used. E, Quantification of Cn-specific activity in hypertrophied and failing hearts.
in each of the samples (Figure 1E). These data confirmed that CnA-specific activity was increased in the hypertrophied but not in the failing hearts. Collectively, these data indicate that the increase in Cn enzymatic activity in failing hearts is due largely to increased expression, whereas in hypertrophied hearts, it is due to both increased expression and increased specific activity.

ERK, p38, and SAPK Activity in Hypertrophied and Failing Human Hearts

We next examined the potential association between MAPK signaling and human hypertrophy and heart failure. The SAPKs were markedly activated (5.3-fold over control) in hearts from patients with advanced heart failure. Lesser but significant activation of the ERKs (3.6-fold) and p38 (2.0-fold) was also found (Figures 2A, 3A, and 4A). Activation of the 3 MAPK pathways was evident, irrespective of whether the underlying disease was ischemia or idiopathic dilated cardiomyopathy (data not shown).

In the hypertrophied heart samples, none of the MAPKs were activated (Figures 2A, 3A, and 4A). Western blotting confirmed the equivalent expression of each of the kinases in normal, hypertrophied, and failing hearts, confirming that increased kinase activity in the failing hearts was due to an increase in specific activity (Figures 2B, 2C, 3B, 3C, 4B, and 4C, bottom panels). We further confirmed the increase in MAPK activity by Western blotting with dual phospho-specific antibodies, which demonstrated increased phosphorylation of SAPK, ERK, and p38 in the samples from failing but not hypertrophied hearts (Figures 2B, 2C, 3B, 3C, 4B, and 4C, top panels). Of note, only the p54 isoform of SAPK, and not the p46 isoform, seemed to be activated in the failing hearts, suggesting differential regulation of these isoforms. Taken together, these data indicate that all 3 MAPK signaling pathways are activated in the failing myocardium but not in the hypertrophied heart.

Akt and GSK-3β Activity in Hypertrophied and Failing Human Hearts

Akt is activated as a downstream consequence of growth factor signaling through phosphoinositide-3 kinases, and
activation of Akt has been associated with protection from apoptosis in cardiac myocytes.9 Because progressive myocyte apoptosis may contribute to heart failure, we examined the activation status of Akt by Western blotting with a phospho-specific antibody. We observed a marked increase in Akt phosphorylation in failing hearts compared with control hearts, but absolute protein levels were invariant (Figure 5C). In hypertrophied hearts, Akt phosphorylation was not significantly increased (Figure 5D).

GSK-3 is a kinase with profound effects on fetal development and tumorigenesis. The activity of GSK-3 is negatively regulated by Akt in many cell types, and inhibiting GSK-3 seems to be critical to the antiapoptotic effects of Akt11 and to the hypertrophic response of cardiomyocytes.13 Consistent with the activation of Akt, we observed a significant inhibition of GSK-3β activity in failing hearts but no inhibition in hypertrophied hearts (Figure 5Aand 5B). The 31% inhibition of GSK-3β in the failing hearts was quite marked considering that IGF-1, a potent inhibitor of GSK-3, produced ~40% inhibition in cardiomyocytes in culture.13 These data indicate that the PI3K/Akt signaling pathway is not activated in hypertrophied hearts but is significantly activated in failing hearts.

We next examined possible mechanisms of Akt activation in human heart failure. Three pro-survival factors that are also implicated in the hypertrophic growth of the heart and that signal via the PI3-kinase/Akt pathway are IGF-1 (which acts through its cognate receptor), neuregulin-1 (which acts through ErbB receptors), and the interleukin-6 family member cardiotrophin-1 (which acts through gp130, a shared signaling subunit of interleukin-6 family receptors).16 To determine the activation state of the receptors for IGF-1 and neuregulin, we immunoprecipitated lysates from normal donor hearts and failing hearts with antibodies to IGF-1Rα and to the ErbB2 receptor and then immunoblotted them with an antibody to phospho-STAT3, one of the

Figure 4. p38 MAPK activity in failing and hypertrophied human hearts. A, Immune complex kinase assays for p38-MAPK were performed on heart lysates from control, hypertrophied, and failing hearts. B, Representative Western blot with phospho-specific p38 antibody (top) and total p38 antibody (bottom) demonstrating an increase in phosphorylation in the failing hearts compared with control (C). C, Western blot for phospho-p38 (top) and total p38 (bottom) demonstrating equivalent phospho-p38 levels in hypertrophied and normal hearts.

Figure 5. PKB/Akt and GSK3β activity in failing and hypertrophied human hearts. A, Immune complex kinase assay for GSK-3β was performed on heart lysates from control, hypertrophied, and failing hearts. GSK3β activity was significantly depressed in failing but not in hypertrophied hearts. B, Western blot with anti-GSK-3β monoclonal antibody confirming equivalent GSK3β protein levels in extracts from control (C), hypertrophied, and failing hearts. C, Western blot with anti phospho-Ser473 PKB/Akt antibody (top) and total PKB/Akt antibody demonstrating significant phosphorylation of Akt in failing hearts compared with controls. D, Western blot for phospho PKB/Akt (top) and total PKB/Akt (bottom) demonstrating equivalent phosphorylation in hypertrophied and control hearts.
major downstream targets of gp130 (data not shown). Although many agonists that are increased in heart failure, including angiotensin II and endothelin-1, can activate Akt, our data suggest that the increased activation of the IGF-1 receptor may in part account for the activation of Akt we observed in advanced heart failure. In contrast, another major prosurvival pathway (signaling via ErbB2) seems to be downregulated, and this may be expected to have an adverse effect on cardiomyocyte survival in the failing heart.

Discussion

Little is known about the intracellular mechanisms that precede human heart failure or the mechanisms that underlie progressive deterioration of function. To date, no studies have reported CnA enzymatic activity in failing human hearts, none have examined the activity of a group of signaling factors in failing hearts, and the activation state of these pathways in hypertrophied human hearts is not known. We postulated that defining an activity profile in both hypertrophied and failing human hearts might provide clues to the mechanisms driving the transition from hypertrophy to heart failure and the progression of heart failure.

Profile of Activity of the Hypertrophied Heart

Our data show striking differences in the activity of signaling pathways between patients with compensated hypertrophy and those with advanced heart failure. Most notably, the Cn pathway seems to be the dominant pathway activated in compensated hypertrophy, and the increased activity is due both to increased expression and to an increase in specific activity. In contrast, we could detect no significant activation of any of the MAPK pathways or the Akt/GSK-3 pathway.

Cn is a calcium-calmodulin–activated intracellular phosphatase that was recently shown to induce cardiac hypertrophy in transgenic mice. There has, however, been ongoing debate concerning the role of Cn in the development of cardiac hypertrophy in response to the physiologically relevant stress of pressure overload. Although the exact role of Cn in the development of acute pressure-overload hypertrophy in experimental animal models remains somewhat unclear, our data suggest that Cn likely does play a role in the maintenance of the hypertrophic phenotype in humans. In contrast, the other signaling pathways we examined do not seem to modulate this phase of the disease. Thus, although various of the MAPK pathways have been clearly implicated in the development of cardiac hypertrophy in experimental animals, it seems from our data that in the compensated phase of chronic hypertrophy, these pathways may not be appropriate targets for therapeutic intervention. In contrast, Cn may be a target for intervention.

Profile of Activity of the Failing Heart

The profile of activity of the various signaling pathways in the failing heart is much more complex than that of the hypertrophied heart. We found significant activation of Cn (although not as marked as in the hypertrophic hearts), marked activation of the SAPKs with lesser activation of the ERKs and p38, and activation of Akt with the corresponding inhibition of its downstream target GSK-3β.

Cn enzymatic activity is increased 1.7-fold in the hearts of patients with failure. Previously, we reported that the content
of Cn complexed with calmodulin was increased in failing human hearts, inferring activation. Although this assay suggests that Cn is in an activated complex with calmodulin, it is uncertain if this accurately reflects enzymatic activity. To address this issue, we directly measured enzymatic phosphatase activity. The enzymatic assay supports our previous conclusions in the failing heart. The data also show that Cn protein content is upregulated in failing hearts, albeit to a lesser extent than that in hypertrophied hearts, and that the increased activity is largely due to this increased expression and not to an increase in specific activity. In contrast, Tsao et al. reported decreased in Cn protein in failing human hearts using 1 vendor source of antibody but increases in Cn using a different antibody. Using the same 2 pan-CnA antibodies, we identified a significant increase in Cn protein content in failing hearts. We also demonstrated a specific increase in CnAβ protein, whereas Cnα was only weakly detected in the adult human heart. Tsao et al. reported decreased levels of CnAβ mRNA in failing hearts, but the probe they used recognizes only CnAβ2, a minor splice form of Cn expressed at low levels in the heart. We think that our Western blotting data, taken together with our activity data, confirm that Cn activity and protein levels are upregulated in heart failure.

For the other signaling pathways, Cook et al. reported that SAPK and p38 phosphorylation were increased in human hearts with failure secondary to advanced CAD. We confirmed the activation of SAPK and p38 in failing hearts due to CAD and extended our observations to include patients with idiopathic dilated cardiomyopathy. Our results indicate that each of the 3 branches of the MAPK signaling pathway are activated in advanced heart failure, irrespective of the cause of the failure.

Activation of the MAPKs in the failing heart may be due to several factors, including increased wall stress (which leads to myocyte stretching), increased levels of neurohormonal mediators of heart failure, or increased circulating cytokines. The activated MAPKs may play various roles in the failing heart, but given the demonstrated role of the SAPKs and, possibly, p38 in the hypertrophic response in vivo, it seems likely that their activation would help normalize wall stress via hypertrophy were it not for the very limited ability of the failing heart to respond.

This report contains the first data associating Akt activation with heart failure. The activation of Akt and consequent inhibition of GSK-3 may protect cells from apoptosis. Therefore, the demonstration of activation of Akt in the hearts of patients with advanced failure is somewhat surprising, because studies have documented apoptosis in these hearts and have postulated that apoptosis may play a role in the progression of heart failure. These data raise questions regarding the roles Akt may play in these hearts. Because Akt can block cell death even after cytochrome c has been released, our data raise the possibility that the widespread aborted apoptosis observed by Narula et al. in the failing heart may be due to Akt activation. In addition, the activation of Akt and the subsequent inhibition of GSK-3 transduce prohypertrophic signals. Finally, Akt regulates GLUT4 translocation, which causes enhanced glucose uptake, and Akt activation may serve to improve energy utilization.

Inhibition of GSK-3β may also have multiple effects in the failing heart. In addition to protecting from apoptosis, GSK-3β phosphorylates the nuclear factors of activated T cells (NF-ATs), which are transcription factors thought to play a role in the hypertrophic response of the heart and in skeletal muscle. This phosphorylation excludes NF-ATs from the nucleus, rendering them inactive. Because Cn dephosphorylates NF-ATs, the activation of Cn and simultaneous inhibition of GSK-3β should provide a potent stimulus to NF-AT nuclear translocation and activation in the failing heart. In contrast, the persistent activation of the SAPKs, which can inhibit Cn/NF-AT interactions, may serve as a check to prevent unstrained activation of the pathway in the failing heart.

Rezvani and Liew recently reported that protein levels (but not mRNA levels) of the transcriptional activator β-catenin were increased in post mortem samples from the hearts of patients with advanced heart disease. Because GSK-3β phosphorylates β-catenin when active, thus targeting it for ubiquitination and degradation, inhibiting GSK-3β activity in the failing heart may be one mechanism of the observed increase in β-catenin expression. The demonstration of altered regulation of a bona fide downstream target of GSK-3β adds additional significance to our findings of the inhibition of GSK-3β in human heart disease.

Study Limitations

Although the 3 groups of patients were matched reasonably well for age and sex, the clinical state of the patients with advanced failure demanded multiple medications and interventions that neither the control nor hypertrophy groups required. Although it is possible that these interventions caused the alterations in signaling in the failing hearts, comparisons within the failure group showed no trends that would suggest a correlation between any medication/device and activation of a signaling pathway. For the pathways, activation was consistent across the entire failure group.

Finally, this study is, by necessity, correlative in nature. However, important insights can still be gained. Our analysis revealed differential regulation of multiple intracellular signaling pathways in the hypertrophied versus the failing hearts, raising the possibility that this differential regulation plays a causal role in the transition from hypertrophy to failure. The data also allow us to form hypotheses concerning signaling pathways that may and may not be appropriate targets for novel therapeutic strategies designed to regress hypertrophy, to influence the transition from compensated to decompensated phenotypes, and to alter the inexorable progression of heart failure. These hypotheses can be readily tested in experimental models of hypertrophy and heart failure.

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References
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