Ras, Akt, and Mechanotransduction in the Cardiac Myocyte

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Abstract—The Ras subfamily of 21-kDa (“small”) guanine nucleotide binding proteins [which includes Ha-Ras, Ki(A)-Ras, Ki(B)-Ras, and N-Ras] is universally important in regulating intracellular signaling events in mammalian cells and controls their growth, proliferation, senescence, differentiation, and survival. These Ras isoforms act as membrane-associated biological switches that transduce signals from transmembrane receptors, thus potentially activating a variety of downstream signaling proteins. These include ultimately two Ser/Thr protein kinase families, the extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt (or protein kinase B). Activation of ERK1/2 has been associated with cardiac myocyte hypertrophy (ie, increased cell size and myofibrillogenesis, with concurrent transcriptional changes to a fetal pattern of gene expression), whereas activation of Akt is associated with the increased protein accretion in hypertrophy. Both ERK1/2 and Akt may promote myocyte survival. In the intact heart in vivo and in primary cultures of cardiac myocytes, mechanical strain induces hypertrophy, a process known as mechanotransduction, which may involve Ras, ERK1/2, and Akt. In this study, general and cardiospecific aspects of the regulation of Ras and Akt will be described. The various mechanisms through which mechanical strain might initiate Ras- or Akt-dependent signaling will be discussed. The overall conclusion is that although an involvement of Ras and Akt in mechanotransduction is likely, more work (particularly focusing on mechanoreception) needs to be undertaken before it is unequivocally established. (Circ Res. 2003;93:1179-1192.)

Key Words: mechanical strain ■ small G proteins ■ protein kinases ■ hypertrophy ■ apoptosis
Ras and Akt are potentially regulated through several classes of transmembrane receptors, including G protein-coupled receptors (GPCRs), receptor protein Tyr kinases (receptor PTKs), and integrins. On agonist engagement, coupled receptors (GPCRs), receptor protein Tyr kinases – classes of transmembrane receptors, including G protein-gated ion channels, activate heterotrimeric G proteins [α(GDP), βγ in their inactive state, dissociating to the biologically active α(GTP) and βγ dimer species on GDP/GTP exchange], whereas receptor PTKs autophosphorylate Tyr residues in their cytoplasmic domains and, in many cases, elicit Tyr phosphorylation of other proteins. In contrast to the unidirectional nature of GPCRs and receptor PTKs, integrin signaling is bidirectional with “outside-in” signaling involving interaction of integrins with the extracellular matrix (ECM) rather than with soluble ligands.

A common feature of Ras and Akt is that both are implicated in hypertrophic growth of the myocardium. Although still actively debated, the prevailing view is that adult mammalian ventricular myocytes are terminally differentiated cells which, although capable of limited karyokinesis, are incapable of cytokinesis. In vivo, the myocardium adapts to a requirement for increased contractile power (eg, during increased hemodynamic load) by increasing its muscle mass, a process brought about predominantly by a true hypertrophy (growth in the absence of cell division) of preexisting cardiac myocytes. The coupling process of overload to hypertrophy is known as mechanotransduction, the molecular nature of which remains poorly understood. Myocardial hypertrophy in vivo is characterized by increases in cell size and sarcomerogenesis over and above that which would be predicted for a given stage of development. Although these changes are brought about, at least in part, by increases in expression of constitutive genes, there are also ancillary transcriptional changes (eg, reexpression of atrial natriuretic factor (ANF) and B-type natriuretic factor (BNP); see the online data supplement) that are associated with adaptive hypertrophy but may also be exhibited by the failing heart. Hypertrophy of myocytes in primary culture can be also induced by mechanical stress (see the online data supplement), as well as by (or through) a plethora of agonists including the GqPCR agonism by endothelin-1 (ET-1), angiotensin II (ANGII), or α-adrenergic stimulation, and by direct pharmacological activators of protein kinase C (PKC), to which GqPCR signaling is also coupled.

### Ras and Its Effectors

#### Activation of Ras

As well as the four closely related proteins generically known as Ras [Ha-Ras, the two alternatively spliced Ki-Ras species, restrictively expressed Ki(A)-Ras and ubiquitously expressed Ki(B)-Ras, and N-Ras, all with a molecular mass of ~21 kDa], the Ras subfamily encompasses several other related proteins that will not be discussed further. Ha-Ras and the Ki-Ras species are essentially entirely (plasma) membrane-bound, although the subcellular localization of N-Ras is less clear. Membrane localization is essential for Ras signaling, and posttranslational lipidation (irreversible farnesylation and methylesterification of Ha-Ras and Ki-Ras, along with reversible palmitoylation of Ha-Ras) contributes significantly to this (see the online data supplement). Furthermore, Ha-Ras and Ki-Ras are differentially localized to membrane microdomains (caveolar or noncaveolar lipid rafts, and “disordered” membrane), and this may account for some of the differential signaling properties of these Ras isoforms.

In its biologically inactive state, Ras is ligated to GDP. Activation of guanine nucleotide exchange factors (GEFs) stimulates exchange of GTP for GDP and produces biologically active Ras.GTP, which then participates in the activation of c-Raf, phosphoinositide 3-OH kinase (PI3K), and Ral.GDS (a GEF for the Ral small G proteins). Innate GTPase activity of Ras, an activity returns Ras.GTP to the “off” state, the GTPase activity being profoundly stimulated by GTPase-activating protein (GAPs).

#### Figure 1. Ras activation cycle. In its “off” state, Ras is ligated to GDP. Activation of guanine nucleotide exchange factors (GEFs) stimulates exchange of GTP for GDP and produces biologically active Ras.GTP, which then participates in the activation of c-Raf, phosphoinositide 3-OH kinase (PI3K), and Ral.GDS (a GEF for the Ral small G proteins). Innate GTPase activity of Ras, an activity returns Ras.GTP to the “off” state, the GTPase activity being profoundly stimulated by GTPase-activating protein (GAPs).
the mechanisms involved in activation of Ras by GPCR ligands are less clear, although these ligands are probably of greater importance in Ras regulation in cardiac myocytes.

**Effectors of Ras.GTP**

The differential lethality of targeted disruption of each of the three Ras genes (the Ki-Ras-null mutation is embryonically lethal in mice\(^1\) but Ha-Ras- or N-Ras-null mice survive as do the double Ha-Ras/N-Ras-null mutants\(^2\)) suggests that there is significant diversity of function between Ras isoforms, and this may be related to differential localization in plasma membrane subdomains.\(^9\) Three effectors of Ras signaling have been identified with certainty (Figure 1)\(^1,13\): the protein kinase c-Raf (and the A-Raf and B-Raf isoforms),\(^13\) PI3K,\(^2,4\) and Rap.GDS (a GEF for the Ras subfamily member, Rap).\(^1\) More recently, phosphoinositide-phospholipase C\(\epsilon\) has been added to this list.\(^14,15\) Whereas the signaling pathways associated with c-Raf or PI3K activation have been studied extensively in the myocardium, those associated with Rap.GDS or related proteins have not and will not be discussed further apart from mentioning that Rap.GDS or related proteins may participate in the hypertrophic response.\(^16\) and that phospholipase C\(\epsilon\) mRNA is expressed relatively abundantly in heart.\(^15\)

**Raf and the Extracellular Signal–Regulated Kinase Cascade**

The Raf family of mitogen-activated protein kinase (MAPK) kinase kinases catalyze the initial step of the 3-membered extracellular signal–regulated kinase 1/2 (ERK1/2) MAPK phosphorylation cascade (Figure 2).\(^1,13,17\) Generally, by modulating transcription factor activity, apoptosis, and other anabolic processes, activation of the ERK1/2 cascade promotes cell growth, division, and survival. The mechanisms that bring about Ras-mediated activation of c-Raf are complex and are not entirely clear.\(^13,17\) In outline, c-Raf is normally cytoplasmic and does not bind to (membrane-associated) Ras.GDP. However, c-Raf has a much higher affinity for Ras.GTP and their interaction translocates c-Raf to the membrane where further interactions and modifications (including phosphorylation of a Ser and a Tyr residue) lead to more complete activation (see the online data supplement).\(^13,17\) Counterbalancing the activating phosphorylations, cAMP-dependent protein kinase\(^17\)–\(^19\) and possibly Akt\(^20\) phosphorylate c-Raf, reducing its activity and/or ability to interact with Ras.GTP. In contrast to c-Raf, B-Raf may be activated by cAMP,\(^13,17\) although this remains controversial.

After its activation, Raf phosphorylates (and activates) the intermediate MAPK kinase (MKK) members of the ERK1/2 cascade on two Ser residues (Figure 2).\(^21\) Two isoforms of MKK are phosphorylated by Raf (MKK1 and MKK2, alternatively known as MEK1 and MEK2, respectively), but whether these differ in their biological functions is unclear. Mutation of these Ser residues to acidic Asp/Glu residues partially mimics phosphorylation and produces a species with greater constitutive activity than the unphosphorylated form. Although this constitutive activity is only a small percentage of the activity of the phosphorylated species, the mutated species is not subject to protein phosphatase–mediated de-

**Effectors of Ras Signaling: PI3K**

As mentioned, Ras.GTP may be involved in activation of the PI3K pathway.\(^1,2\) PI3K phosphorylates membrane 3-OH phos-
phosphoinositides, producing the membrane-localized second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃] when PtdIns(4,5)P₂ is substrate (Figure 4). PI3K is a heterodimer consisting of a catalytic subunit (molecular mass ≈110 kDa) and a regulatory subunit (see the online data supplement). There are three species of catalytic subunit (p110α, p110β, and p110δ) in the class IA PI3Ks, and these heterodimerize with one of the three species of regulatory subunit (p85α, p85β, or p55). For class IA PI3K, the only catalytic subunit so far identified is p110γ, and the only regulatory subunit is p101. All PI3K catalytic subunits possess a lipid kinase domain, which may also display a limited ability to phosphorylate Ser/Thr residues in proteins, and a Ras interaction domain. Interactions of PI3K regulatory subunits with membrane proteins following receptor stimulation place PI3Ks in the plane of the membrane. For class IA PI3Ks, these interactions are with auto-phosphorylated phospho-Tyr residues in receptor PTKs and their associated docking proteins; for class IB PI3K, these interactions are with heterotrimeric G protein βγ dimers. Thus, PI3K is placed in proximity to the Ras-GTP formed in a receptor PTK- or GPCR-dependent manner and its membrane substrates. Many of the signaling functions of PtdIns(3,4,5)P₃ depend on its recognition and binding of proteins containing pleckstrin homology (PH) domains. Other PH domain proteins may bind selectively to other phosphoinositides such as PtdIns(3,4)P₂ or PtdIns(4,5)P₂. PtdIns(3,4,5)P₃-dependent signaling is terminated by hydrolysis either to reform PtdIns(4,5)P₂ by 3′-lipid phosphatases such as PTEN (a tumor suppressor, mutation of which can predispose to malignancies), or to PtdIns(3,4)P₂ by 5′-lipid phosphatases such as the SHIPs.

Regulation of Ras Activation in the Heart
As reviewed earlier, previous work in isolated myocytes or in transgenic mice has suggested that V12Ha-Ras induces a hypertrophic phenotype in the myocardium, although some inconsistencies in the in vivo response in particular have been detected. Conversely, inhibitory N17Ha-Ras reduces agonist-stimulated ANP expression in isolated myocytes. As might be predicted given the importance of membrane localization of Ras, farnesyl transferase inhibitors attenuate some of the morphological and transcriptional changes associated with norepinephrine- or ET-1–induced myocyte hypertrophy. Inhibition of hydroxymethylglutarlyl CoA reductase, the rate-controlling step for polyprenoid and cholesterol biosynthesis, by the statins is a less-specific way of reducing Ras farnesylation, but statins reduce stimulation of protein accumulation by ANGII in cardiac myocytes, and importantly, the inhibition can be prevented by mevalonate. However, polyprenoids are involved in the modification of other small G proteins other than Ras, so these results must be interpreted with caution.

In isolated myocytes, ET-1, the pharmacological α-adrenergic agonist phenylephrine (PE), and phorbol 12-myristate 13-acetate (PMA), a phorbol ester that directly activates certain PKC isoforms, each maximally activate Ras rapidly (30 seconds to 1 minute), as does ANGII and...
stimulation of a Ras.GEF or inhibition of a Ras.GAP, the former being the more likely to produce the rapid activation of Ras occurring in the cardiac myocyte.29 Probably the best-characterized Ras.GEF is Sos,10 and Sos protein is expressed in cardiac myocytes.30 What is less clear is whether Sos is involved in the activation of Ras by ET-1, etc, in these cells. By binding to their GqPCRs, ET-1 and PE stimulate the activity of Gq protein–regulated phosphoinositide phospholipase Cβ and hydrolysis of PtdIns(4,5)P2 to (membrane-localized) diacylglycerol (DAG),31 the physiological activator of conventional and novel PKC isoforms (Figure 3),28 whose action is mimicked pharmacologically by suitable phorbol esters. In addition to activating Ras, another feature shared by PMA, ET-1, and PE is that they rapidly (<30 seconds) translocate PKCε from the soluble to the particulate fraction (interpreted as indicating PKCε activation) in cardiac myocytes,32,33 and there is some evidence that PKCε participates in the activation of Ras in cardiac myocytes.29 Because of the rapidity of the response, it seems likely that, if PKC signals to Ras, the interconnection between them cannot be complex, although the mechanism is not understood. Interestingly, the existence of a “signaling cassette” complex between PKCε, N-Ras, and c-Raf has been demonstrated in fibroblasts,34 and there is evidence that PKCε-containing “signaling cassettes” are present in the cardiac myocyte.35 However, the DAG-dependent PKCs may not represent the only intracellular DAG receptors that are able to activate Ras. Thus, Ras.GEFs that bind DAG and phorbol esters (eg, Ca2+- and phorbol ester–binding Ras.GEFs such as Ras.GRP) have been identified,36 although it should be pointed out that Ras.GRP appears to be primarily neuronal and may not be of significance in the myocardium.

In terms of transcriptional regulation in the cardiac myocyte, it is worth mentioning that ERK1/2 instigate phosphorylation and activation of the transcription factors including Elk-137 and GATA-438 (Figure 2), and that Ras activates the NF-AT3 (NF-ATc4) transcription factor in an ERK1/2 cascade-dependent manner.39 In addition, the transcriptional coactivators CBP and p300 are activated through ERK1/2.40 All these transcription factors have been implicated in the regulation of gene expression in cardiac myocyte hypertrophy. Elk-1 has been identified as an important factor in transcription of the immediate early gene c-fos,24 which has long been known to be rapidly expressed on hypertrophic stimulation, whereas GATA-4 regulates transcription of hypertrophic index genes including ANF and BNP genes.41 In addition to stimulating transcription in their own right, NF-ATs cooperate with GATA-4 to increase expression of the BNP gene.42

We and others have proposed that the ERK1/2 cascade represents an important signaling pathway in myocyte hypertrophy.43 Convincing evidence of an in vivo role has recently been presented,44 with transgenic mice expressing constitutively activated MKK1 in a cardiac myocyte-directed manner exhibiting a largely adaptive hypertrophy in which the hearts enlarge, “fetal” genes are reexpressed (see the online data supplement), and dP/dt max is increased (although there is a typical negative lusitropic effect). Additionally, myocytes isolated from these hearts show increased resistance to apoptosis. However, ERK1/2 activities are not increased in the hypertrophied hearts of transgenic mice cardioselectively expressing V12Ha-Ras, although the activities of c-Jun N-terminal kinase, a stress-responsive MAPK, are.45 Although this finding has been interpreted as implicating JNKs, and not ERK1/2, in promotion of Ras-mediated hypertrophy, an alternative view is that the activation of JNKs is an effect of the ensuing Ras-induced cardiac failure rather than an initiator of adaptive hypertrophy.

Much attention has been focused recently on the “triple-membrane-passing signal” pathway of Ras-dependent signaling.46 In this pathway, GqPCR stimulation causes phosphorylation and transactivation of the EGF receptor, with subsequent activation of the ERK1/2 cascade and increased c-fos expression. ET-1 and other GqPCR agonists transactivate the EGF receptor in cardiac myocytes, and this may play an important role in myocardial hypertrophy.47-49 The mechanism involves matrix metalloproteinases, cleavage of extracellular surface-bound pro–heparin-binding EGF and heparin-binding-EGF shedding, followed by activation of the EGF receptor.47,49,50 The pathway(s) connecting GqPCR activation to metalloproteinase stimulation is obscure and may variously involve PKCα, Ca2+, reactive oxygen species (ROS), and nonreceptor PTKs.50 Other possible pathways of ERK1/2 cascade activation include the endocytotic β-arrestin–dependent pathway (see the online data supplement), although this has been studied mainly in connection with GsPCR/GiPCR signaling; however, it has also been implicated in signaling from GqPCRs such as the ET_{A} and AT_{1} receptors, although its relevance to the cardiac myocyte has not been fully explored.

**Regulation of Akt (PKB)**

Research into Akt is very active currently and has been summarized in recent excellent reviews,2,3 (see also the online data supplement) and only basic salient features are summarized here. Like Ras, Akt is encoded by three genes in mammals, with Akt1/PKBα being the most widely expressed and best studied. The differing phenotypes displayed by mice with targeted genomic disruption of Akt1 or Akt 2 (both of which are viable) not only imply that Akt1 and Akt2 may fulfill different roles in vivo, but also that there may be a limited ability of Akt1 to substitute for Akt2, and vice versa.51,52 As described earlier, activation of PI3K increases the membrane content of PtdIns(3,4,5)P3, and this leads to activation of Akt (Figure 4). There are two strands to this. Akt contains an N-terminal PH domain that binds to PtdIns(3,4,5)P3, and formation of PtdIns(3,4,5)P3 translocates inactive Akt from the cytoplasm to the membrane. However,
in spite of some reports to the contrary, the general view is that binding of Akt to PtdIns(3,4,5)P₃ is not sufficient to activate the kinase. Another PH domain kinase, PtdIns(3,4,5)-dependent kinase 1 (PDK1), has greater affinity for PtdIns(3,4,5)P₃ than Akt, and additionally binds to PtdIns(3,4)P₂, some of which is produced by SHIP-mediated hydrolysis of PtdIns(3,4,5)P₃. Thus, a proportion of the cell’s complement of PDK1 is constitutively bound to the membrane, and, in contrast to Akt, binding of PDK1 to these 3-phosphoinositides does activate the kinase. The translocation of Akt to the membrane thus juxtaposes the two kinases, and Akt is phosphorylated on Thr³⁰⁸ in Akt1 (or an equivalent Thr residue in Akt2 and Akt3). However, full activation of Akt1 also requires phosphorylation of Ser⁴⁷³. The kinase responsible for Akt1(Ser⁴⁷³) phosphorylation is not clear, although experiments using a PDK1⁻/⁻ embryonic stem cell line suggest that it is not PDK1. It has been suggested that Akt1(phospho-Thr³⁰⁸) autophosphorylates or transphosphorylates Akt1(Ser⁴⁷³), although recently a distinct PDK2 activity may mediate some of the effects originally ascribed to Akt because an overlap in recognition of substrate oligopeptide motifs (for details of Akt specificity, see the online data supplement). Recently, phosphorylation at Thr³⁰⁸ has been described. Importantly, Akt is not the only substrate of PDK1, which is directly involved in the phosphorylation of other AGC family kinases such as the 70-kDa ribosomal protein S6 kinases (p70S6Ks). In some cases, these kinases may mediate some of the effects originally ascribed to Akt because an overlap in recognition of substrate oligopeptide motifs (for details of Akt specificity, see the online data supplement). The problem of overlapping substrate specificities has been compounded by the fact that, until recently, there were no small molecule inhibitors of Akt, only of PI3K (wortmannin, LY294002).

**Biological Roles of Akt**

A multiplicity of biological effects have been ascribed to Akt (Figure 4). By inhibiting apoptosis at multiple points, Akt promotes cell survival. The regulation of apoptosis and its significance in the cardiac myocyte has recently been reviewed in depth and will not be described in detail. Akt modulates carbohydrate metabolism through phosphorylation and inhibition of glycogen synthase kinase 3 (GSK3), although the inhibition of GSK3 has wider consequences in the heart. It may also regulate fuel metabolism in heart by activating 6-phosphofructo-2-kinase, which produces the allosteric activator of the 6-phospho-fructokinase step of glycolysis, fructose 2,6-bisphosphate, and through (a probably indirect) inhibition of AMP-regulated protein kinase. The role of Akt in cardiac fuel metabolism will not be discussed further, but it has been reviewed recently. Akt regulates protein synthesis through mechanisms involving the activation of the mammalian target-of-rapamycin, eukaryotic initiation factor 4E-binding proteins (4E-BPs), and p70S6K (see the online data supplement). This aspect of Akt signaling is perhaps particularly important in considering the outcome of expression constitutively active Akt in the cardiac myocytes of transgenic mice, but like cardiac protein synthesis generally, its importance is underestimated. Finally, Akt phosphorylates and activates endothelial nitric oxide synthase, which is important in regulation of cardiac function and is present in both cardiac myocytes and cardiac endothelial cells.

**Akt Enhances Cell Survival by Inhibition of Apoptosis**

Akt increases cell survival through transcription-independent and -dependent mechanisms. Bcl-2 proteins play critical positive and negative roles in the regulation of outer mitochondrial membrane integrity, which is crucial for cell survival. Bcl-2 itself and Bcl-Xl are antiapoptotic proteins associated with this compartment and probably to prevent proapoptotic Bcl-2 family proteins such as Bax and Bak from forming poorly characterized pores through which cytochrome c and other proteins are released into the cytoplasm, thereby promoting apoptosis. Membrane integrity is maintained by formation of heterodimers of Bax or Bak with Bcl-2 or Bcl-Xl. BH3-only Bcl-2 family proteins such as Bad, Bid, and Bim interact with Bcl-2/Bcl-Xl through their BH3 domains and, by removing the antiapoptotic influence of Bcl-2/Bcl-Xl on Bax and Bak, promote pore formation. However, phosphorylation of Bcl(X) and its consequent sequestration by 14-3-3 proteins (which recognize phosphoser residues in sequence-specific contexts) tethers Bad in the cytoplasm, thus diminishing its proapoptotic potential. Although this phosphorylation was originally attributed to Akt, more recent evidence has indicated that it is p70S6K, which recognizes the same consensus sequence as Akt, that phosphorylates Bcl(X) and its consequent sequestration by 14-3-3 proteins (which recognize phosphoser residues in sequence-specific contexts) tethers Bad in the cytoplasm, thus diminishing its proapoptotic potential. Although this phosphorylation was originally attributed to Akt, more recent evidence has indicated that it is p70S6K, which recognizes the same consensus sequence as Akt, that phosphorylates Bcl(X) and its consequent sequestration by 14-3-3 proteins (which recognize phosphoser residues in sequence-specific contexts) tethers Bad in the cytoplasm, thus diminishing its proapoptotic potential. Although this phosphorylation was originally attributed to Akt, more recent evidence has indicated that it is p70S6K, which recognizes the same consensus sequence as Akt, that phosphorylates Bcl(X) and its consequent sequestration by 14-3-3 proteins (which recognize phosphoser residues in sequence-specific contexts) tethers Bad in the cytoplasm, thus diminishing its proapoptotic potential. Although this phosphorylation was originally attributed to Akt, more recent evidence has indicated that it is p70S6K, which recognizes the same consensus sequence as Akt, that phosphorylates Bcl(X) and its consequent sequestration by 14-3-3 proteins (which recognize phosphoser residues in sequence-specific contexts) tethers Bad in the cytoplasm, thus diminishing its proapoptotic potential. Although this phosphorylation was originally attributed to Akt, more recent evidence has indicated that it is p70S6K, which recognizes the same consensus sequence as Akt, that phosphorylates Bcl(X) and its consequent sequestration by 14-3-3 proteins (which recognize phosphoser residues in sequence-specific contexts) tethers Bad in the cytoplasm, thus diminishing its proapoptotic potential. Although this phosphorylation was originally attributed to Akt, more recent evidence has indicated that it is p70S6K, which recognizes the same consensus sequence as Akt, that phosphorylates Bcl(X) and its consequent sequestration by 14-3-3 proteins (which recognize phosphoser residues in sequence-specific contexts) tethers Bad in the cytoplasm, thus diminishing its proapoptotic potential. Although this phosphorylation was originally attributed to Akt, more recent evidence has indicated that it is p70S6K, which recognizes the same consensus sequence as Akt, that phosphorylates Bcl(X) and its consequent sequestration by 14-3-3 proteins (which recognize phosphoser residues in sequence-specific contexts) tethers Bad in the cytoplasm, thus diminishing its proapoptotic potential.
in its promoter region and its transcription is upregulated by the (dephosphorylated) FOXO, FKHR1. In addition, FOXOs may play a role in promoting the expression of proapoptotic Bim while reducing expression of antiapoptotic Bcl-2 family genes.

There are at least three less well-characterized transcription-dependent pathways for Akt-mediated cell survival, namely those dependent on the NF-κB, CREB, or p53 transcription factors. Nuclear translocation of NF-κB, which is normally retained in the cytoplasm in unstimulated cells through sequestration by IκB, increases expression of genes encoding inhibitor of apoptosis proteins and (as first shown by Zong et al) antiapoptotic Bcl-2 family members. Akt-mediated activation of NF-κB may involve stimulation of pathways leading to IκB phosphorylation (which results in proteosomal IκB degradation) or more direct effects on NF-κB itself. The transcription factor CREB is a substrate for cAMP-dependent protein kinase and other protein kinases, and its transactivating activity is increased by phosphorylation of Ser33. This residue is also phosphorylated by Akt, and the Akt-dependent upregulation of expression of Bcl-2 may be mediated through phosphorylation of CREB. Akt may also regulate the transcription-dependent proapoptotic activity of p53 by promoting the translocation of Mdm2, a p53 ubiquitin ligase, to the nucleus, leading to proteosomal degradation of p53 and increased cell survival.

**Glycogen Synthase Kinase 3: An Important Effector of Akt Signaling**

GSK3 was originally identified as a Ser/Thr protein kinase that phosphorylates and inhibits glycogen synthase, the enzyme that catalyzes the rate-controlling step in glycogen synthesis, although the influence of GSK3 is now recognized to be considerably wider. The regulation of GSK3 has been reviewed recently (see also the online data supplement), and will only be described in outline in this review. GSK3 represents a major substrate of Akt, with phosphorylation of Ser19 in GSK3α or Ser21 in GSK3β being inhibitory (this is one pathway through which insulin promotes glycogen accumulation), and mutation of these residues to Ala produces constitutively active kinases. These Ser residues are also phosphorylated by other protein kinases such as p90RSKs and p70S6Ks and thus represent intersections allowing cross-talk between signaling pathways. In addition, GSK3 is regulated through the Akt-independent, developmentally important Wnt pathway, although the insulin-sensitive pool of GSK3 and Wnt pathway–associated pool of GSK3 appear to be separate. The Wnt pathway regulates the stability of β-catenin, a protein involved directly in both transcriptional regulation and cell adhesion, and Wnt-mediated inhibition of GSK3 causes accumulation of unphosphorylated β-catenin, which is less susceptible to degradation than the phosphorylated species.

**Akt and GSK3 in the Myocardium: Regulation of Cell Survival and Growth**

**Regulation of Akt in the Myocardium**

The potential therapeutic benefit of activation of the PI3K/Akt pathway in the heart is manifest. However, there have been relatively few studies of the regulation of Akt and most have used only phosphorylation of the Akt(Ser473)-like site as the sole criterion of activation. In cardiac myocytes, insulin and H2O2 induce the greatest degree of Akt(Ser473) phosphorylation; serum is a moderately powerful agonist, but PMA, ET-1, and PE are at best only weak agonists. The differing potencies of insulin or PE in stimulating Akt(Ser473) phosphorylation are also reflected in enzymic activity of Akt. Ras.GTP loading on its own is clearly insufficient to activate the PI3K-Akt pathway in cardiac myocytes (because Ras is strongly activated by PMA, ET-1 and PE in cardiac myocytes), and additional signals (eg, receptor PTK phosphorylation) are presumably required. Cytokine signaling [the interleukin-6–related cytokines, leukemia inhibitory factor, and cardiotoxin-1 (CT-1)] also stimulates the phosphorylation/activity of Akt (and PI3K activity and p70S6K phosphorylation) in cardiac myocytes, and erythropoietin has recently been identified as another factor that activates Akt. The cytoprotective properties of insulin and cytokines are discussed in greater detail in the following section.

The activation of Akt by H2O2 in cardiac myocytes is something of an anomaly, although it has been detected in other cells. H2O2 may be insulin-mimetic in this regard through increasing Tyr phosphorylation of the insulin receptor or other receptor PTKs by inhibition of protein tyrosine phosphatases. However, H2O2-mediated Akt phosphorylation, unlike insulin, does not result in the anticipated phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) in myocytes, a phosphorylation that promotes protein synthesis. In fact, unlike insulin, relatively low concentrations of H2O2 strongly inhibit the overall rates of protein synthesis and are cytotoxic to myocytes. In spite of these findings, ROS such as H2O2 have been implicated in the promotion of growth and the stimulation of expression of some genes in cardiac myocytes. The biological reasons for the ability of H2O2 to activate Akt are unclear. They may genuinely relate to growth promotion or may represent an endogenous attempt to increase survival.

**Akt and Myocardial Protection**

Apoptotic cells have been detected in the myocardium in a variety of pathological situations, although estimates of the frequency differ dramatically. Although there has been disagreement about the significance of apoptosis in myocardial pathologies, the elegant experiments of Kitsis and coworkers have demonstrated that even relatively low levels of initiator caspase 8 activity induce myocardial failure. That activation of Akt is directly protective in whole heart and isolated cardiac myocytes is now well-documented (see review and online data supplement for details of the original articles), the suggestion strongly being that this is due to a reduction in apoptosis. This is likely to be one facet of the cardioprotective and/or antiapoptotic effects of insulin and IGF1 (see, for example, Aikawa et al; see the online data supplement for further references). In addition, Akt and its effectors have been implicated in the protection mediated by ischemic preconditioning. Overexpression of constitutively activated Gaq activates phospholipase Cβ and this results in...
PtdIns(4,5)P₂ hydrolysis. In the myocardium, although this potentially activates the PKC-Ras-ERK1/2 pathway, it also depletes the membrane of the PI3K substrate and reduces Akt activation. This is an interesting result, because it may provide an explanation for some inconsistencies. The balance between activation of signaling pathways and between the overall biological result could be shifted depending on the membrane PtdIns(4,5)P₂ content. It could even explain why some investigators (including ourselves) have failed to see activation of Akt with powerful GqPCR agonists such as ET-1.

Cytokine-based signaling may also involve Akt. CT-1 is both hypertrophic and is a potent survival factor in cardiac myocytes. It stimulates phosphorylation of Bad(Ser136) through a PI3K-dependent pathway (presumably through Akt or p70S6K), and increases cardiac myocyte survival after serum starvation. These antiapoptotic effects of CT-1 are also in part dependent on the ERK1/2 cascade, which is activated by this ligand and NF-κB. Leukemia inhibitory factor is also cytotoxic in the cardiac myocyte, inhibiting doxorubicin-induced apoptosis and promoting phosphorylation of Bad through PI3K. The Bad phosphorylation site was not specified in this study, but was presumably Bad(Ser136). Bad(Ser112) undergoes antiapoptotic phosphorylation in a manner that appears to be largely dependent on the ERK1/2 cascade and the p90RSKs and such a pathway exists in cardiac myocytes. Thus, inhibition of Bad-mediated apoptosis may be regulated through both PI3K/Akt and the ERK1/2 cascade. This agrees with work showing that, although the protective effects of IGF1 against hypoxia in the myocardium, although this does not promote sarcomerogenesis or ANF expression, is ischemic preconditioning through a PI3K-dependent path-

Inhibition of GSK3β by phosphorylation of Ser9 through the PI3K/Akt pathway promotes ET-1- or β-adrenergic–mediated hypertrophy of cultured cardiac myocytes. Furthermore, transfection or adenoviral transfer of GSK3β(Ser9→Ala) inhibits ET-1- or isoproterenol-induced hypertrophy as assessed by sarcomereogenesis or ANF protein expression. Inhibition was blocked by Li⁺, which, at concentrations of 10 mmol/L, inhibit GSK3β (but probably not many other protein kinases) by about 40%.

GSK3 and Myocardial Hypertrophy and Protection

Hypertrophic agonism may be mediated by the cardiac myocyte-restricted transcription factor GSK3β. Wnt-independent stabilization of β-catenin could be relevant to hypertrophy (at least in cultured cells) in terms of the promotion of intercellular contacts.

GSK3 phosphorylates other transcription factors in addition to β-catenin. These include the c-Jun DNA binding domain (inhibiting DNA binding and attenuating c-Jun-dependent transcription), and NF-AT2 (also known as NF-ATC or NF-ATc1). Phosphorylation of NF-ATs promotes their exclusion from the nucleus, and accordingly, ET-1 promotes nuclear translocation of NF-AT (possibly NF-AT2) in myocytes in a manner that is inhibited by expression of GSK3β(Ser9→Ala). Somewhat confusingly, ET-1 also translocates (presumably inactivated?) GSK3β to the nucleus, the function of which is unclear. The GSK3-mediated stimulation of ANF expression by β-adrenergic agonism may be mediated by the cardiac myocyte-restricted transcription factor GATA-4. Activated GSK3 promotes nuclear export of GATA-4 in cardiac myocytes through the nuclear exportin, Crm1, although it is not clear whether GSK3 phosphorylates GATA-4 directly. Of ancillary interest is the finding that β-adrenergic agonism promotes the association of NF-AT2 and GATA-4 in cardiac myocytes.
overall conclusion is that inhibition of GSK3 should increase binding of c-Jun to its consensus sequences, and promote nuclear entry/retention of NF-AT2 and GATA-4, thus stimulating gene expression from promoters sensitive to these transcription factors.

Manipulation of Akt and GSK3 Activities in Transgenic Mice

Akt can be constitutively activated by membrane-targeting (eg, introduction of a c-Src myristoylation motif), or by mutation of Thr308 and Ser473 to Asp, and three groups have reported the phenotypes of mice expressing such species in a cardiac myocyte–directed manner.114–116 The prediction that the phenotype should resemble that for mice expressing a constitutively activated PI3K117 is largely borne out. The hearts are larger as a result of an increase cardiac myocyte mean size,114–116 and there is resistance to ischemia/reperfusion injury.116 There were some changes in contractile properties, although these were somewhat variable between the mouse lines. However, where detected, any enhancement of cardiac contraction-relaxation may be the result of changes in Ca2+ movements mediated by upregulation of SERCA2a.118 From the signal transduction standpoint, p70S6K was phosphorylated as predicted but, surprisingly, GSK3β phosphorylation was not universally detected,114–116 although this could relate to time of sampling. When GSK3β phosphorylation was detected, nuclear translocation of GATA-4 was also observed.114 The mammalian target-of-rapamycin (see the online data supplement) was necessary for the expression of the Akt1(Thr308→Asp,Ser473→Asp) phenotype and p70S6K activation.116 Gene expression profiling by DNA microarrays of one Akt transgenic line did not reveal any changes in the expression of hypertrophic marker genes, although expression of genes that might contribute to the effects of Akt on cardiomyocyte survival, metabolism, and growth was affected.119 Overall, the phenotypes of the activated Akt transgensics suggest that Akt has two roles: (1) to coordinate the myocardial mass with maturational and nutritional signals120 and (2) to cytoprotect.6

Transgenic mice have also been used to study the role of GSK3β in the heart.121 Cardiac myocyte–directed expression of GSK3β(Ser3→Ala) alone has little effect on heart weight/body weight ratio, but in combination with expression of constitutively activated calcineurin, GSK3β(Ser3→Ala) reduced calcineurin-induced increases in heart weight/body weight ratio and reduced accumulation of NF-ATs in the nucleus.121 Similarly, GSK3β(Ser3→Ala) reduces the increase in relative heart weight induced by β-adrenergic stimulation or aortic constriction. From the point of view of transcriptional criteria of hypertrophy, results were confusing. Although the calcineurin-induced increase in β-myosin heavy chain expression was reduced by GSK3β(Ser3→Ala), the expression of ANF or BNP was increased.121

The outcome of activation of the receptor-mediated [Fas ligand (FasL)/Fas] apoptosis pathway in myocytes is ambiguous,57 but may be rationalized to some extent by its actions on GSK3. Although FasL is reportedly proapoptotic in cultured myocytes,122 the phenotype of mice expressing FasL in a cardiac myocyte–directed manner is one of cardiac inflammation and mild hypertrophy, rather than frank apoptosis.123 This result is somewhat unexpected (forced FasL expression would be predicted to be proapoptotic), although it has recently been suggested that FasL inhibits GSK3β in isolated neonatal cardiac myocytes through the PI3K pathway and thus promotes their hypertrophy.124 In vivo, mice lacking a functional Fas receptor display a cardiomyopathic rather than a hypertrophic response after aortic constriction and GSK3β was not inhibited (unlike the wild-type situation).124 However, mice lacking functional FasL did mount a hypertrophic response after aortic constriction, possibly because Fas receptor transactivation from other receptors is a feature of the hypertrophic response.124

Involvement of Ras and Akt in Cardiac Mechanotransduction

The topic of mechanotransduction in the heart has been fully reviewed (see also the online data supplement),7 and the focus here will primarily relate to involvement of Ras and Akt in this process. The commonly used experimental models (see the online data supplement) ex vivo are cyclical or static strain of myocytes attached a deformable matrix (which demonstrably induces hypertrophy) and perfused hearts. In vivo, constriction of the (thoracic) aorta is probably the most frequently used intervention. Given the established functions of Ras and Akt, it is inherently likely that they, at least in part, mediate mechanotransductional growth, although direct evidence is relatively exiguous. Likewise, direct evidence that they are activated during mechanotransduction is relatively sparse, and it is necessary largely to rely on surrogate indicators, such as activation of the ERK1/2 cascade for Ras, and phosphorylation of p70S6K or GSK3 for Akt. An alternative strategy is to establish the participation in mechanotransduction of factors known to activate Ras or Akt, thus peptide growth factors, ROS, and ECM signaling will be discussed. Mechanosensitive ion channels will not be discussed as there is currently no evidence of which the author is aware that their activity affects Ras or Akt.

With respect to direct evidence of Ras and Akt activation in mechanotransduction, there is only one report to my knowledge that strain increases Ras-GTP loading (in statically stretched myocytes).125 The activation of Ras in vivo has not been studied. Phosphorylation of Akt (and of eNOS) in an LY294002-inhibited manner in matrix-embedded stretched myocytes has been demonstrated,126 and increased phosphorylation of Akt (and of GSK3β) has been detected in acute (10-minute) pressure-overload in mouse hearts.127 Furthermore, work in the early 1990s showed that strain activates a multiplicity of signaling molecules that are known to lie either upstream or downstream of Ras, including phospholipases, PKC, ERK1/2, and p90RSK.125 In vivo, acute or chronic pressure overload activates ERK1/2 and p90RSK,127–129 and consistent with a role for Gq/11PCRs in hypertrophy, conditional targeted genomic disruption in the heart of the α subunits of the heterotrimeric G proteins, Gq and G11,130 or cardiac myocyte-specific expression of a peptide that interferes with interaction of GqPCRs with Gq,131 reduces pressure-overload hypertrophy. Disruption of the G protein α
subunit genes would be expected to prevent agonist-dependent activation of Ras.29

Local Release of Peptides and Other Species That Activate Ras or Akt
Conditioned medium from mechanically strained cardiac myocytes stimulates hypertrophic responses in “naïve” myocytes,123 suggesting that local release of hypertrophic factors is important. A variety of approaches both ex vivo and in vivo (see the online data supplement) support a role for (local) release of ANGII or endothelins. As mentioned above, both ET-1 and ANGII activate Ras in cardiac myocytes,29,30 although there is little evidence that they are effective activators of Akt in these cells.79 Metalloproteinase-mediated shedding of HB-EGF may be important in both pressure-overload hypertrophy and in GqPCR agonist-induced hypertrophy.45 As mentioned earlier, activation of the EGFR receptor potentially stimulates Ras.GTP loading and Akt activation. All of these correlative data certainly implicate Ras and possibly implicate Akt. Less explored is any involvement of gpl30-related signaling. Although gp130-linked signaling is implicated in cardiac hypertrophy ex vivo,96 possibly through the involvement of ERK1/296 and Akt,82,83 results of experiments in which expression of CT-1 or leukemia inhibitory factor has been examined in vivo under overload conditions have been equivocal.101,102 However, cardiomyocytic overexpression of a dominant-negative gp130 reduced the magnitude of pressure-overload hypertrophy in transgenic mice.129 As mentioned, pressure-overload of hearts of transgenic mice in which the gp130 gene had been cardiomyocyte-specifically disrupted produced a dilated cardiomyopathy rather than an adaptive hypertrophy,100 raising the possibility that an adaptive response would have manifested itself had the gp130 gene not been disrupted.

Although ROS are cytotoxic at higher concentrations,87 they may directly promote growth of cardiac myocytes under more benign circumstances.88 From the signaling standpoint, H2O2 activates MAPK cascades and the PI3K/Akt pathway in cultured myocytes.79,113,114 although it is not known whether Ras is involved. As described in the online data supplement, there is evidence that mechanical strain increases ROS production in cardiac myocytes.

Extracellular Matrix–Based Signaling
More general details of ECM-based and focal adhesion–based signaling are given in the online data supplement. In vivo, the adult cardiac myocyte interacts both with other myocytes and with the ECM.135 The attachment of the myocyte to the ECM and any subsequent stretching of the myocyte during hemodynamic overload may stimulate growth. One cardiac myocyte–specific locus for interaction with the ECM is the costamere. Costameres are protein complexes that lie adjacent to the Z lines of subsarcolemmal myofibrils and contain cytoskeletal proteins such as vinculin, β1-integrin, and other proteins,135 including the recently identified β1-integrin–interacting protein, melusin.136 Interactions between integrins and the ECM are important in myocyte growth and hypertrophy.137 Targeted disruption the β1-integrin gene renders mice intolerant to pressure-overload and leads, in the longer term, to dilated cardiomyopathy even in the unloaded situation.138 Disruption of the melusin gene prevents pressure-overload hypertrophy in vivo, but does not prevent hypertrophy induced by subpressor doses of ANGII or PE.127 Parenthetically, the phosphorylation of Akt or GSK3β was decreased in melusin-null mice compared with wild-type after short- (10 minutes) or long-term (7 days) pressure overload.127 Interaction between laminins of the ECM, the dystrophin-dystroglycan-sarcoglycan complex, and myofibrillar structures represents another example of myocyte-ECM interaction in vivo,139 and the interaction may influence nuclear events, possibly being transmitted by the cytoskeletal/intermediate filament protein, desmin.140

Like many cells, cardiac myocytes in culture form focal adhesions where they attach to an ECM substrate such as fibronectin.135 The simple attachment of myocytes to a fibronectin matrix induces activation of focal adhesion–related signaling pathways,141 and myocyte growth and hypertrophy.141,142 However, the relationship of focal adhesions in cultured myocytes to myocyte-ECM interactions in vivo are not understood in detail.135 Focal adhesion signaling proteins such as the focal adhesion kinase (FAK), the docking protein p130Cas, and the adaptor/scaffold protein paxillin are located in the region of the Z line in isolated neonatal cardiac myocytes and may play a role in the maintenance of sarcomere structure.143,144 The concept is emerging that FAK is not so much a protein kinase as a self-regulating docking protein providing a scaffold that activates other signaling molecules. Myocyte strain or pressure overload ex vivo activates focal adhesion signaling (eg, increased Tyr phosphorylation of FAK).145–147 and activation of focal adhesion signaling is known to lead to stimulation of multiple signaling pathways including the Ras-ERK1/2 and PI3K/Akt pathways in other cells.148 Integrin/focal complex–based signaling has also been implicated in the pressure-overloaded ventricle in vivo,149,150 although, in this situation, the cellular heterogeneity of the heart is a complicating factor and it is not clear whether this signaling pathway is sufficient on its own to induce the hypertrophic response. Interestingly, the pattern of Tyr phosphorylation of FAK was not consistent with a role in pressure-overload–induced LV hypertrophy in vivo, although the pattern of expression and Tyr phosphorylation of the p125FAK-related protein kinase, PYK2, was more so.151 The overall conclusion is that ECM-based signaling, possibly by acting through focal adhesions, is a facet of strain-induced hypertrophy.

Do Ras and Akt Participate in Overload-Induced Hypertrophy In Vivo? Overall Conclusions
Although it seems likely that Ras and Akt participate in cardiac mechanotransduction, this still remains to be definitively established. Probably the best direct test would be to use transgenic mice in which the various Ras or Akt genes are inactivated or cardioselectively express suitable dominant-negative Ras or Akt mutants. Targeted genomic disruption of Ha-Ras and/or N-Ras genes, or of the Akt1 or Akt2 genes is not lethal in mice, although, phenotypically, the mice may differ from their wild-type littermates.1,2,51,52 Mice that selectively express dominant-negative Akt in their cardiac myo-
cytes have also been derived. These animals should be suitable for direct experimentation on the effects of hemodynamic overload, although no experiments of this nature have been reported.

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Ras, Akt, and Mechanotransduction in the Cardiac Myocyte
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Background

Ras and Akt were each identified some years ago as virally-encoded oncogenes (for details, see Reference 1, and References 2,3, respectively). These particular oncogenes are counterparts of normal cellular genes that have mutated in such a way that the encoded oncoproteins are constitutively active, thus removing the restraint of normal cellular regulation. Both oncoproteins (especially Ras) are potentially important in human tumorigenesis, and they represent viable targets for therapeutic intervention. Likewise, the protein kinases Raf and Src (which are also mentioned in the article) were first identified as viral oncogenes, with their wild-type cellular counterparts being important in the regulation of a variety of biological processes in untransformed cells.

Changes in Gene Expression Coincident with Ventricular Hypertrophy

As mentioned in the main article, the prevailing view is that adult mammalian ventricular myocytes are terminally-differentiated cells which, although capable of limited karyokinesis, are incapable of cytokinesis. However, this topic is still actively debated.\(^4\) Thus, the increase in contractile capacity in ventricular hypertrophy is brought about by the growth of pre-existing myocytes rather than through myocyte division and this response, at least in part involves changes in gene expression.\(^5\) Genes that are rapidly (within 1 h) but transiently expressed following the exposure of cultured cardiac myocytes or hearts in vivo to hypertrophic interventions are c-\textit{jun}, c-\textit{fos} and egr-1.\(^6\) These are 'immediate early genes' which are defined as genes which can
be expressed as mRNA in the absence of de novo protein synthesis, indicating that the various factors required for their transcription are present constitutively. The c-*jun* and c-*fos* genes (which together encode proteins which regulate transcription through AP-1 consensus sequences in promoter regions)\(^7\) and *egr*-1 (a zinc-finger transcription factor encoding gene)\(^8\) are rapidly upregulated,\(^6\) and they may encode proteins which may participate in the initiation of the transcriptional changes involved in the hypertrophic response. Other transcription factors which are constitutively-expressed and may be involved in the hypertrophic response\(^9\) are the GATA\(^{10-12}\) and NF-AT\(^{13}\) factors, the latter which at least in part mediate the calcineurin pathway of hypertrophy.\(^{14}\) The collaboration of GATA with other transcription factors is important, for example, in the regulation of the ANF gene in myocytes.\(^{15}\) Another rapidly-expressed gene is that which encodes B-type natriuretic peptide (BNP), but unlike c-*jun* etc., expression is maintained for longer.\(^{16}\) Over a more chronic time course, there is re-initiation of a 'fetal' program of gene expression, i.e. genes which are only normally expressed in the fetal heart are re-expressed. In the rat or mouse heart, these include genes encoding atrial natriuretic factor (ANF), skeletal muscle α-actin and β-myosin heavy chain (β-MHC). Although these changes are not necessarily specific to these rodent species, it should be noted that β-MHC expression differs between species. In the rat and mouse, α-MHC normally predominates. Expression of α-MHC is reduced in hypertrophy with expression of β-MHC correspondingly increasing.\(^6\) Conversely, in man and rabbit, β-MHC is the major isoform expressed.\(^{17-19}\) In the rabbit, thyrotoxicosis produces a hypertrophy characterized by a shift towards expression of α-MHC, whereas pressure overload induces a hypertrophy with a shift to β-MHC expression.\(^{17}\) Although α-MHC constitutes only about 5% of the total MHC complement in the normal human left ventricle, a decrease in its relative expression can
be seen in heart failure,\textsuperscript{19} though any changes in β-MHC expression would not be detectable against the high pre-existing background. The biological basis for increased ANF and BNP expression may be related to diuresis and natriuresis particularly in hypertensive states, whereas the changes in actin and MHC expression are probably related to the increased efficiency of contraction observed in the hypertrophied myocardium.\textsuperscript{20} More recently, ANF and BNP have been reported to exert an antihypertrophic effect directly on the myocardium through cyclic GMP-based signaling (the ANF and BNP transmembrane receptors have intracellular guanyl cyclase activity) and their increased expression may thus represent a negative feed-back loop.\textsuperscript{21} In addition to the 'fetal' program, there are changes in the expression of constitutively-expressed genes. With respect to contractile proteins and the increased sarcomerogenesis, cardiac muscle α-actin and ventricular myosin light chain expression is increased.\textsuperscript{6} There are also changes in the expression of protein that regulate Ca\textsuperscript{2+} mobilization. Thus expression of sarcoplasmic (endoplasmic) reticulum Ca\textsuperscript{2+} ATPase, which is responsible for re-uptake of Ca\textsuperscript{2+} into the sarcoplasmic reticulum, is probably reduced (at least at the mRNA, if not the protein, level).\textsuperscript{22} Similarly, the abundance of the sarcoplasmic reticulum Ca\textsuperscript{2+} release channel (the ryanodine receptor type 2, responsible for Ca\textsuperscript{2+}-initiated Ca\textsuperscript{2+} release from the sarcoplasmic reticulum) is reduced.\textsuperscript{23} There may also be changes in the expression of the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger, but there is little consensus as to the direction as yet.\textsuperscript{24} The various changes in these proteins which regulate Ca\textsuperscript{2+} movements probably contribute to the altered contractile properties (slowed rates of contraction and relaxation) of hypertrophied myocytes.

Cellular Localization and Differential Signaling Functions of Ras
Interactions with intracellular membranes are frequently a facet of small G protein signaling, and the interaction of Ras with and partitioning to specific regions in the plasma membrane are particularly important in Ras-dependent signaling. Ha-Ras, Ki(A)-Ras, and Ki(B)-Ras are essentially entirely (plasma) membrane-bound. The subcellular localization of N-Ras is less clear and it may also be present in mitochondrial membranes. Plasma membrane localization of Ras is achieved by irreversible S-farnesylation by farnesyl transferases and subsequent methylesterification of the C-terminal Cys-residue in Ras. However, these modifications are insufficient to ensure membrane interaction. In the case of Ha-Ras and N-Ras, reversible palmitoylation of one or two nearby C-terminal Cys-residues reinforces the interaction. Palmitoylation may increase partition of Ha-Ras to 'lipid rafts', membrane regions which are rich in cholesterol, sphingolipids, phospholipids with saturated side-chains and, in some cases, caveolin. In the case of Ki(B)-Ras, a polybasic region in the C-terminal region is important in membrane interaction because it interacts with negatively-charged polar head groups of membrane phospholipids in 'disordered' membrane regions. Indeed, its migration to the membrane may instigate formation of regions of 'disordered' membrane structure. Furthermore, the partitioning of Ras to different regions in the membrane and movement between these regions may account for the differential efficacy with which Ras isoforms signal to their downstream effectors. As emphasized in the review, this is a very active area of research at the moment (though little has been done in cardiac myocytes) and further details can be found elsewhere.

The sequence of lipidation is as follows. The initial Ras translation product contains a C-terminal -Cys-Ali-Ali-Xaa-COOH sequence where Ali- is an amino acid
with an aliphatic side chain and Xaa is Ser- or Met-. The thiol group of Cys is post-
translationally farnesylated by farnesyl transferase with farnesyl pyrophosphate
(otherwise encountered as an intermediate of cholesterol synthesis) as donor. The C-
terminal tripeptide is then cleaved and the carboxyl group of the (now C-terminal) Cys
is methylated with S-adenosyl-methionine as the methyl donor. These 2 modifications
are irreversible and indeed have been used experimentally (by the introduction of a C-
terminal Cys-Ala-Ala-Xaa sequence) to target non-Ras proteins to the membrane. In 3
of 4 Ras species [Ki(B)-Ras is the exception], reversible palmitoylation of other Cys
residues in the C-terminal region (particularly the Cys at the -2 position from the
modified C-terminal Cys) probably reinforces the strength of interaction of Ras with
membranes. Because the lipid modifications of Ras and the associated membrane-
targeting are necessary for it to fulfill its signaling functions, they may represent a point
for therapeutic intervention in cancer.33,34

Activation of Ras by Receptor Protein Tyrosine Kinases

Extracellular ligand-driven autophosphorylation of Tyr-residues in receptor
protein tyrosine kinases [receptor PTKs, such as those for insulin, insulin-like growth
factor-1, epidermal growth factor, etc.] creates binding sites for docking proteins (eg.
Shc, insulin receptor substrates, the Grb2-associated binders Gab1 and Gab2) and/or
adaptor proteins (eg. Grb2).28,35,36 Docking and adaptor proteins recognize specific
phospho-Tyr-containing motifs through either their SH2 or PTB domains.37 Docking
proteins also become Tyr-phosphorylated creating further binding sites for adaptor
proteins and other SH2/PTB-containing proteins. Thus the Grb2 adapter protein
contains 2 SH3 domains separated by a single SH2 domain. Grb2 is associated with a
Pro-rich domain in the Ras guanine nucleotide exchange factor, Sos through its more N-terminal SH3 domain,\textsuperscript{37} and the complex is cytoplasmic. On cell activation, the ensuing Grb2-receptor PTK (or docking protein) interaction brings the Grb2-Sos complex to the plasma membrane and thus juxtaposes Sos and membrane-bound Ras.GDP, leading to GDP/GTP exchange and generation of Ras.GTP.

Ras Mutants

Ras residues Gly\textsuperscript{12} and Gln\textsuperscript{61} are critical in inactivating Ras and their mutation (eg. in V12Ha-Ras, in which Gly\textsuperscript{12} is mutated to Val-) reduces the innate and GTPase activating protein- (GAP-) stimulated GTPase activity, thus rendering Ras constitutively-active. In an in vivo setting, these mutations are potentially oncogenic, though, in an experimental setting, expression of the oncoproteins is valuable in implicating participation of Ras in cellular processes. In contrast, 'inhibitory' or 'dominant-negative' mutants of Ras (eg. N17Ha-Ras, in which Ser\textsuperscript{17} is mutated to Asn) have also been extensively to implicate Ras in signaling pathways. This mutant acts by ligating Ras.GEFs with high affinity, thus preventing their interaction with endogenous Ras.GDP.\textsuperscript{38}

Activation of c-Raf
c-Raf is normally cytoplasmic but the interaction of c-Raf with Ras.GTP results in its translocation to the membrane where further modifications and interactions lead to full activation.\textsuperscript{39,40} Phosphorylation at the membrane of c-Raf on Ser\textsuperscript{338} and Tyr\textsuperscript{341} by poorly-characterized Ser-/Thr- and Tyr-specific protein kinases is strongly associated with activation.\textsuperscript{39,40} Current candidate kinases for Ser\textsuperscript{338} phosphorylation include p21-activated kinase (PAK), and in particular, PAK3,\textsuperscript{41,42} which are activated by the Rac1 and cdc42 small G proteins through the phosphatidylinositol 3-OH kinase pathway (PI3K).\textsuperscript{42-45} However, the involvement of PI3K and PAK3 in c-Raf(Ser\textsuperscript{338}) phosphorylation has recently been called into question.\textsuperscript{46} Non-receptor PTKs of the Src family\textsuperscript{47} are candidate kinases for the Tyr\textsuperscript{341} phosphorylation.\textsuperscript{48-50} Equally, it should be recognized that Ser\textsuperscript{338} and Tyr\textsuperscript{341} are not the only sites in c-Raf known to be reversibly phosphorylated. In contrast to phosphorylation of Ser\textsuperscript{338} and Tyr\textsuperscript{341}, cAMP-dependent protein kinase (PKA) interferes with Ras.GTP/c-Raf interaction by phosphorylating Ser\textsuperscript{43} in the c-Raf/Ras interaction domain,\textsuperscript{40} though this has also been questioned.\textsuperscript{51} Additional PKA sites (Ser\textsuperscript{233}, Ser\textsuperscript{259}, Ser\textsuperscript{621}) may also be involved in the regulation of c-Raf activity.\textsuperscript{52,53} Phosphorylation of Ser\textsuperscript{233} and Ser\textsuperscript{259} promotes association of c-Raf with 14-3-3 proteins, thus preventing its activation by Ras.GTP.\textsuperscript{52,53} The situation with respect to Ser\textsuperscript{621} is more equivocal,\textsuperscript{52,54,55} with one suggestion being that phosphorylation of this residue is important in retaining c-Raf in an inactive state through 14-3-3-protein binding before its activation by Ras.GTP, but the same phosphorylation is involved in maintaining c-Raf in its active conformation with altered 14-3-3 protein binding following the multi-step activation process.\textsuperscript{39} Somewhat anomalously in view of its growth promoting properties, Akt has also been reported to inhibit c-Raf activity by phosphorylating Ser\textsuperscript{259}.\textsuperscript{56,57} These differential effects of phosphorylation on the activity of c-Raf are important to bear in mind when designing experiments that investigate c-
Raf phosphorylation. Other factors which may positively regulate c-Raf activation include its interaction with (membrane) phospholipids, such as phosphatidylserine\textsuperscript{58,59} and phosphatidic acid,\textsuperscript{39,60} and interactions with other proteins such as the kinase suppressor of Ras (KSR). Although KSR was originally identified as a positive mediator of Ras signaling that possessed many structural features associated with protein kinases, it is now thought not to be a protein kinase.\textsuperscript{61} It is now considered to be a scaffolding protein for the extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascade that increases the proximity of the ERK1/2 cascade components.\textsuperscript{61}

The $\beta$-arrestin pathway of ERK1/2 activation

Binding of agonists to the $\beta_2$-adrenoceptor activates G protein coupled-receptor kinase 2 (GRK2, also known as $\beta$-adrenergic receptor kinase 1).\textsuperscript{62-64} This is achieved through the membrane-associated $\beta\gamma$ dimers formed after GDP/GTP exchange and dissociation of heterotrimeric Gs and/or Gi proteins [(\textit{G}DGP),$\beta\gamma$] into $\textit{G}$TP and $\beta\gamma$ on activation of the $\beta_2$-adrenoceptor. GRK2 binds to the $\beta\gamma$ dimers and phosphorylates the $\beta_1$-adrenoceptors. These phosphorylations create binding sites for $\beta$-arrestin, thus targeting the $\beta_2$-adrenoceptor to clathrin-coated pits in the membrane, with subsequent dynamin-dependent endocytosis of the membrane-associated clathrin-coated vesicles that are formed. The $\beta_2$-adrenoceptor is subsequently dephosphorylated and recycled to the plasma membrane. However, $\beta$-arrestin in clathrin-coated pits and/or vesicles may also act as a scaffolding protein for signaling molecules such as c-Src and members of the ERK1/2 cascade, leading to their activation. The model further proposes that the formation of endosomal vesicles from cell surface clathrin-coated vesicles may play a role in targeting of ERK1/2 to the cytoplasm (rather than the nucleus).
Activation of Phosphatidylinositol 3-OH Kinases

The regulatory subunits of class 1A PI3Ks (p85α, p85β, and p55) contain SH2 domains and a Pro-rich region. The former recognize phospho-Tyr containing oligopeptide sequences in proteins (e.g., in receptor PTKs or their associated adaptor proteins) and the latter may also be involved in protein-protein interactions. p85α and p85β contain an additional Pro-rich domain, an SH3 domain (which generally recognize Pro-rich regions in proteins), and a 'Rho-GAP' domain (a domain that is found in GAPs for small G proteins of the Rho subfamily). For class 1A PI3Ks, the autoposphorylation of membrane-bound receptor PTKs or, more likely, the Tyr-phosphorylation of membrane-associated adaptor/docking proteins by receptor PTKs leads to the binding of PI3K regulatory subunits through their SH2 domains. Proteins which may be important in this regard include the insulin receptor substrates (phosphorylated by the insulin receptor), and Gab1/2 which are phosphorylated by receptor PTKs in a Grb2-dependent manner. These interactions place the class 1A PI3Ks in the plane of the membrane in proximity to Ras.GTP formed in an receptor PTK-dependent manner and in proximity to the PI3K substrate, phosphatidylinositol 4,5-bisphosphate. Thus class 1A PI3K activity in the plane of the membrane and the membrane content of phosphatidylinositol 3,4,5-trisphosphate are increased. In a similar manner, binding of class 1B PI3K to the membrane bound βγ dimers formed from heterotrimeric G protein dissociation [α(GDP),βγ→α(GTP) + βγ] following GPCR activation places that PI3K in the plane of the membrane adjacent to any Ras.GTP formed on GPCR activation and again the membrane content of phosphatidylinositol 3,4,5-trisphosphate is increased.
The Oligopeptide Motif Phosphorylated by Akt

In addition to the reviews cited in the main article, several further valuable reviews are available. Other reviews describe PDK1, the controversy surrounding phosphorylation of Ser by PDK2, and the regulation of cell growth by Akt in more detail. Following activation, Akt phosphorylates Ser/Thr residues in proteins containing a recognition sequence initially characterized as Arg-Xaa-Arg-Xaa-Xaa-(Ser-/Thr-) [RXRXX(S/T)] with Xaa- or X representing any amino acid. It is important to realize that this motif is also recognized by other 'AGC' kinases such as the 90 kDa ribosomal protein S6 kinases, the 70 kDa ribosomal protein S6 kinases (p70S6Ks), and the serum- or glucocorticoid-induced kinases. Using an orientated peptide library approach, the optimum sequence requirements for Akt were further refined to Arg-Lys-Arg-Xaa-Arg-Thr-Tyr-Ser-Phe-Gly, with the Ser-residue being the most favored for phosphorylation. It is now possible to search proteins using a protein motif-scoring algorithm (available at http://scansite.mit.edu) for consensus sequences for Akt (and several other protein kinases) and obtain a score for their susceptibilities to phosphorylation. However, it should be noted that susceptibility to phosphorylation by Akt is dependent on factors other than simple primary sequence, including the existence of reciprocal docking sites.

Regulation of Protein Synthesis by Akt

Activation of Akt leads to stimulation of translational protein synthesis. This involves a complex and still unclear signaling network that is mediated significantly
through the mammalian target-of-rapamycin (mTOR, so called because it is inhibited by the macrolide antibiotic rapamycin through the FK506-binding protein) and p70S6Ks. Activation of protein synthesis initiation is dependent on the phosphorylation of the eukaryotic initiation factor (eIF) 4E-binding proteins (4E-BPs), whereas translation of mRNA species encoding ribosomal proteins is enhanced by phosphorylation of ribosomal protein S6. However, it should be recognized that, in addition to the PI3K/Akt pathway, the ERK1/2 cascade may also have a significant input into the stimulation of protein synthesis in cardiac myocytes.

Species of mRNA that are synthesized in the nucleus contain a 5' 7-methyl-GTP (m7GTP) cap. The m7GTP cap in mRNAs is recognized by eIF4E in a trimeric complex with the eIF4G scaffolding protein and the eIF4A RNA helicase. This trimeric complex is known collectively as eIF4F. The 5'-untranslated regions of mRNA species contain regions of secondary structure (double-stranded regions) which prevent formation of the 43S ribosomal initiation complex. In conjunction with eIF4B, the eIF4F complex unwinds the regions of secondary structure allowing eIF4G-mediated binding of the 40S (small) ribosomal subunit. When the 40S ribosomal subunit and its associated factors (including Met-charged initiator tRNA) bind to the now single-stranded 5'-untranslated region of the mRNA, the ribosome scans the mRNA in a 5' to 3' direction until it recognizes an AUG initiation codon in a favored sequence context (a Kozak sequence). The initiation factors then dissociate, and the large 60S ribosomal subunit associates with the 40S ribosome/mRNA complex, allowing translation to begin. eIF4E is thus crucial in the initiation of translation. It is prevented from forming the eIF4F complex by its binding of the 4E-BPs. However, phosphorylation of 4E-BPs dissociates the eIF4E/4E-BP complex allowing cap-dependent translation to begin.
The p70S6Ks phosphorylate the small ribosomal protein S6, which favors translation of mRNAs that contain 5' terminal oligopyrimidine (TOP) tracts (though this has been challenged recently). The mRNAs encoding the proteins of the ribosomal translational machinery are distinguished by the presence of TOP tracts, thus activation of p70S6K potentially increases translational capacity by stimulating synthesis of ribosomal proteins.

The phosphorylation of 4E-BPs and the phosphorylation and activation of p70S6Ks is thus crucial to the stimulation of protein synthesis. mTOR seems central to this though the process is not fully understood. mTOR is a large (290 kDa) protein that probably possesses Ser-/Thr- protein kinase activity towards 4E-BP and p70S6K, stimulation of which is initiated by Akt. In addition, there appears to a separate and direct input of the 3-phosphoinositide-dependent kinase 1 (PDK1) into p70S6K, with the phosphorylation of p70S6K by mTOR 'priming' it for phosphorylation by PDK1. It will be recalled that PDK1 also phosphorylates and activates Akt, and thus the full phosphorylation of p70S6Ks require a 'circular' signaling loop.

How does activation of Akt stimulate the protein kinase activity of mTOR? The tuberous sclerosis complex proteins TSC1 (hamartin) and TSC2 (tuberin) appear critical to this activation. TSC1 and TSC2 are tumor suppressor genes and mutations in either can lead to tuberous sclerosis, a disease which is characterized by the development of usually-benign tumors (harmatomas) in a variety of organs (including the heart). Harmatomas are groups of dysplastic, disorganized cells with growth potential within an organ. TSC1 and TSC2 are large proteins (130 and 198 kDa, respectively) that appear to act in concert as TSC1/TSC2 heterodimers to
negatively-regulate mTOR. It appears that phosphorylation of TSC2 by Akt relieves this inhibition, possibly by promoting binding of 14-3-3 proteins. However, the activation of p70S6K mediated by TSC-dependent Akt signaling has also been reported to be mTOR-independent. The ERK1/2 cascade may also instigate phosphorylation of TSC2 at sites which are distinct from the Akt sites. It is not clear how the TSC1/TSC2 complex inhibits mTOR. TSC2 possesses a GAP domain and has GAP activity towards the small G protein, Rheb (Ras homology enriched in brain), and this appears to reduce phosphorylation of both 4E-BP and p70S6K, presumably through mTOR. Phosphorylation of TSC2 presumably reduces its GAP activity and thus stimulates GTP-loading of Rheb, which, in a way that is not understood, stimulates mTOR activity.

Phosphorylation of Bad

The topic of cardiac myocyte apoptosis has been reviewed extensively. Bcl proteins play important roles in the regulation of apoptosis, and may be either antiapoptotic or proapoptotic. There are 2 consensus phosphorylation sequences for Akt in the proapoptotic Bcl-2 protein Bad. Bad, Ser112 and Ser136 (using the mouse numbering system), with the major site for phosphorylation by Akt being initially identified as Ser136. This antiapoptotic phosphorylation promotes retention of Bad in the cytoplasm through its sequestration by 14-3-3 proteins. The situation may not be this simple. First, p70S6K, rather than Akt, may be responsible for phosphorylation of Bad(Ser136). Second, there are other antiapoptotic phosphorylation sites in Bad including Ser155, a site in the BH3 domain which is a substrate for PKA. One hypothesis is that phosphorylation of the mitochondrial Bad/Bcl-XL complex on
Bad(Ser\textsuperscript{130}) and the ensuing binding of 14-3-3 proteins leads to phosphorylation of Bad(Ser\textsuperscript{155}), promoting dissociation of the Bad/Bcl-X\textsubscript{L} complex and translocation of Bad to the cytoplasm.\textsuperscript{103} Bad(Ser\textsuperscript{117}) is also subject to antiapoptotic phosphorylation in a manner that appears to be largely dependent on the activation of the ERK1/2 cascade and the p90RSKs,\textsuperscript{107-110} and we have recently demonstrated the existence of such a pathway in cardiac myocytes.\textsuperscript{111} This also points to the possibility that Bad-mediated protection against apoptosis exerted by Ras may have two arms: through activation of PI3K/Akt and through the ERK1/2 cascade. Very recently, Bad(Ser\textsuperscript{178}) has also been identified as a antiapoptotic phosphorylation site by an unidentified protein kinase,\textsuperscript{112} but the significance of this finding is not yet clear.

Phosphorylation of Forkhead Transcription Factors

One of the best established involves phosphorylation of Forkhead box transcription factors of class O subfamily (FOXOs),\textsuperscript{113,114} which includes FKHR, FKHRL1 and AFX in man. Each contains 3 RXRXX(S/T) consensus sequences for phosphorylation by Akt,\textsuperscript{115-118} though the relative importance of each of the 3 sites in terms of their susceptibility to phosphorylation by Akt and in terms of the biological regulation of FOXOs is unclear. Originally, phosphorylation of one or more of these sites (Thr\textsuperscript{32} and Ser\textsuperscript{253} in FKHRL1) was thought to result in the retention of FOXOs in the cytoplasm through sequestration by 14-3-3 proteins, thus preventing them from activating transcription,\textsuperscript{116} though this has been questioned.\textsuperscript{119} Phosphorylation of Thr\textsuperscript{24} in FKHR is critical for interaction with 14-3-3 proteins, but rendering Ser\textsuperscript{256} resistant to phosphorylation by mutation to Ala indirectly blocks 14-3-3 protein binding by preventing the phosphorylation of Thr\textsuperscript{24}.\textsuperscript{119} Furthermore, phosphorylation of Ser\textsuperscript{256}
may mask a nuclear localization signal, thus preventing translocation to this compartment.

Phosphorylation of and by GSK3

In addition to the review cited in the main article, further valuable reviews on GSK3 are available. GSK3 is inactivated by Akt-mediated phosphorylation of a Ser-residues in the N-terminal region (Ser in GSK3α, Ser in GSK3β), though there is also an Akt-independent phosphorylation site on a Tyr-residue (Tyr in GSK3α, Tyr in GSK3β) catalyzed by an ill-defined PTK. Very recent evidence has suggested that this phosphorylation may represent an intramolecular autophosphorylation event. This Tyr-phosphorylation appears to be required for GSK3 activity, though it may be permissive rather than regulatory, as well as possibly stabilizing the enzyme. GSK3 phosphorylates up to 4 Ser-residues in glycogen synthase in a 'relay pattern' within repeating -Ser-Xaa-Xaa-Xaa- Ser- motifs and the initial phosphorylation of the more N-terminal Ser-residue by GSK3 depends on the more C-terminal Ser-residue being phosphorylated. In the case of the most C-terminal Ser- in the relay, a 'priming' phosphorylation is necessary and this is mediated by casein kinase II. GSK3 is then able to phosphorylate the Ser- lying at the -4 position relative to the casein kinase II site. Subsequently, the Ser-residue phosphorylated by GSK3 acts as the priming phosphorylation of the next Ser-residue lying at the -4 position by GSK3. The finding that a priming phosphorylation is necessary for phosphorylation of glycogen synthase by GSK3 is also relevant to the inactivation of GSK3 by phosphorylation of Ser in GSK3α or Ser in GSK3β which creates an inhibitory internal pseudosubstrate site by mimicking the priming phosphorylation, but the
residue at -4 is not liable to phosphorylation. The necessity for an analogous relay of priming phosphorylations may be a characteristic of other GSK3 substrates.

Akt and Cardioprotection

Numerous studies have shown that Akt is cardioprotective against a multiplicity of potentially damaging events both in vivo and ex vivo. The activation of Akt by insulin or insulin-like growth factor-1 is probably a major facet of the cardioprotection afforded by these receptor PTK ligands and, has been mentioned, involves the suppression of pro-apoptotic pathways. The review by Matsui et al. has been cited in the main article. Further references on the effects of Akt itself include References 127-129, and references on the phosphoinositide 3'-kinase/Akt-mediated cardioprotection or myocyte protection afforded by insulin or insulin-like growth factor-1 include References 130-133.

Ex vivo and in vivo Models Used in the Study of Mechanotransduction

In addition to Reference 134, further reviews describing cardiac mechanotransduction are available. The most common experimental manoeuvre to elicit a hemodynamic-overload myocardial hypertrophy is to impose a pressure overload on the left ventricle of experimental animals by constricting the (thoracic) aorta, simulating the effects of pathological hypertension. There are additionally a wide variety of other models of pressure overload. Volume-overload hypertrophy can be induced by exercise and again there is a wide range of models available including
enforced swimming or treadmill exercise. However, the perceived necessity for enforced exercise is misplaced. Mice provided with a cage wheel will voluntarily run significant distances and a volume-overload hypertrophy results.\textsuperscript{138} Ex vivo, acute changes in the activation of signalling pathways by increased afterload can be studied in the perfused heart (retrograde or anterograde perfusion mode) as can changes in the rate of protein synthesis and the very early effects on patterns of gene transcription, but this preparation does not survive for a sufficient length of time for changes in myocyte size to be detectable. Isolated myocytes from neonatal rat hearts can, when attached to deformable membranes coated with a suitable substrate (eg. collagen, fibronectin), be 'stretched' either statically or phasically (usually at about 0.5 - 1 Hz) (see, for example, References 139-141. ('Stretch' is an imprecise term from a physical viewpoint and the term strain, which has a precise physical meaning, is preferred. Strain is the proportional deformation induced in a body by the application of a stress, which is a force.) Increasingly sophisticated technology has allowed biaxially uniform strain to be combined with electrical pacing of myocytes, thus imposing cellular deformation during a specific phase of the cardiac cycle. This allows the signaling pathways modulated by 'volume-overload' (diastolic) strain or 'pressure-overload' (systolic) strain to be studied separately.\textsuperscript{142} Myocytes can also be implanted in deformable matrices and stretched (see, for example, Reference 143). It is assumed that the strained myocyte ex vivo simulates hemodynamic overloading in vivo, but this is not entirely justified. For example, rates of stretching of rat myocytes ex vivo (usually about 0.5 - 1 Hz) are always less than the 5-6 Hz in vivo, since it is not possible to achieve such rates ex vivo.

Furthermore, increased force of contraction in the whole heart is mediated by an increase in intracellular Ca\textsuperscript{2+} (Ca\textsubscript{i}\textsuperscript{2+}) transient and/or by an increase in the sensitivity of the myofibrillar ATPase to Ca\textsuperscript{2+}. The changes observed in Ca\textsuperscript{2+} sensitivity and Ca\textsuperscript{2+}
movements during an imposed stretch may well differ significantly from those seen in vivo.\textsuperscript{144} However, the strained myocyte is still perhaps the best controlled experimental system and the myocytes survive for a sufficient period to allow changes in gene expression (which parallel those in vivo) to be detected.\textsuperscript{135,139-141} Increased contractile activity can also be induced either by increasing cell density to cause increased spontaneous contraction,\textsuperscript{145} or induced by electrical stimulation at about 3 Hz,\textsuperscript{146} though again it is not clear to which in vivo processes these are analogous. The participation of autocrine/paracrine loops involving ET-1 and ANGII has been described in the main article. Thus a variety of approaches have been used ex vivo to implicate ANGII or ET-1 including ET\textsubscript{A} or ANGII type 1a/1b (AT1a/b) receptor antagonists,\textsuperscript{147-149} radioimmuno assay of released ET-1 or ANGII,\textsuperscript{147,150} and demonstration of increased expression of ET-1 precursor mRNA.\textsuperscript{150} Further indirect evidence comes from in vivo studies of hemodynamic overload in which hypertrophy has been reduced independently of hemodynamic load by ET\textsubscript{A}- or AT1-receptor antagonists,\textsuperscript{151,152} or ACE inhibitors.\textsuperscript{152,153}

Integrins and Extracellular Matrix-based Signaling: Background Information

Integrins are ubiquitous transmembrane glycoproteins which interact with extracellular matrix (ECM) molecules such as laminin, fibronectin or collagen.\textsuperscript{154} A variety of integrins are expressed in the cardiac myocyte.\textsuperscript{155} Although involved in cell adhesion, integrins also possess bidirectional signaling functions, transmitting extracellular signals to the intracellular compartment and vice versa. Integrins are heterodimers of an \( \alpha \) and a \( \beta \) subunit, both of which are represented by multiple species (24 potential \( \alpha \) and 9 potential \( \beta \) subunits have been detected in the human genome).
Pairing of $\alpha$ and $\beta$ subunits is not promiscuous with only certain combinations permitted. Both $\alpha$ and $\beta$ subunits comprise a large extracellular domain, a transmembrane domain and a short intracellular domain. Interactions of the intracellular domain of the integrin $\beta$ subunits with other proteins ($\alpha$-actinin, talin, vinculin) provide a connection between the ECM and the cytoskeleton. Integrins are selective in terms of their interaction with the ECM. Thus, $\alpha_\epsilon\beta_1$ integrin is a major receptor for fibronectin, recognizing an -Arg-Gly-Asp- (-RGD-) containing sequence.\textsuperscript{156,157} Consequently, oligopeptides containing RGD sequences interfere with the binding of $\alpha_\epsilon\beta_1$ integrin to fibronectin (though they also interfere with certain other integrin-ECM interactions) and are hence valuable in the study of integrin biology.

When cultured cells are plated on a suitable ECM substrate, engagement of integrins with their extracellular ligands and/or integrin clustering stimulates the formation of 'focal adhesions' or 'focal complexes'.\textsuperscript{154,158,159} These are complexes of numerous proteins including cytoskeletal proteins, non-receptor PTKs [eg. 125 kDa focal adhesion kinase (FAK),\textsuperscript{160-163} the p125-FAK-related kinase, PYK2,\textsuperscript{164} c-Src kinase (CSK) and c-Src\textsuperscript{165}], protein Ser-/Thr- kinases [eg. protein kinase C, p21-activated kinase (PAK)\textsuperscript{43,44}], docking proteins (eg. p130Cas\textsuperscript{166}), adapter/scaffolding proteins (eg. paxillin, itself a non-receptor PTK substrate\textsuperscript{167,168}), small G protein modulators, and protein and lipid phosphatases (eg. protein tyrosine phosphatases and PTEN\textsuperscript{169,170}). The formation of focal adhesions leads to the activation of intracellular signaling including activation of the ERK1/2 cascade.\textsuperscript{171}

Two Models for Attachment-dependent Activation of Intracellular Signaling Pathways
Model 1: The more intricate details of focal adhesion-mediated regulation of intracellular signaling pathways may differ, but the overall scheme described here is generally applicable. The formation of focal adhesions induces the autophosphorylation of FAK(Tyr\textsuperscript{397}), a phosphorylation that may require an intact actin cytoskeleton and activation of the small G protein, Rho. FAK(phospho-Tyr\textsuperscript{397}) then recruits membrane-bound c-Src by binding through the c-Src SH2 domain.\textsuperscript{47,165} In catalytically-inactive c-Src, this SH2 domain is ligated intramolecularly to c-Src(phospho-Tyr\textsuperscript{527}) (a phosphorylation catalyzed by CSK), but competition from FAK(phospho-Tyr\textsuperscript{397}) disrupts this interaction. This not only 'relaxes' the c-Src structure leading to dephosphorylation of c-Src(phospho-Tyr\textsuperscript{527}), but also stimulates autophosphorylation of c-Src(Tyr\textsuperscript{416}) and activation of c-Src. c-Src then phosphorylates FAK(Tyr\textsuperscript{925}), thus creating a docking site for Grb2 of the Grb2-Sos complex. In a manner analogous to receptor PTK signaling,\textsuperscript{28,36} these events place Sos in the proximity of membrane-bound Ras, which is activated by the GEF activity of Sos, leading to activation of the ERK1/2 cascade.

Model 2:\textsuperscript{172} Association of caveolin-1 with defined integrin \(\alpha\)-subunit family members (eg. \(\alpha_1\)-integrin) promotes the formation of a complex with the c-Src-family member c-Fyn, and the docking protein, Shc.\textsuperscript{37,173} Shc is then Tyr-phosphorylated by c-Fyn and recruits the Grb2-Sos complex, consequently activating the ERK1/2 cascade possibly through caveolin-associated Ha-Ras. However, cardiac myocytes are probably devoid of caveolin-1, though they do express the related caveolin-3 and are major site of expression of this isoform in the heart.\textsuperscript{174} It is thus difficult to assess the role of this signaling pathway in the cardiac myocyte, though, perhaps the finding that targeted disruption of the caveolin-3 gene in mice leads to a form of decompensated cardiac
hypertrophy (ventricular wall thickening with increased fibrosis and reduced fractional shortening) and hyperactivation of the ERK1/2 cascade is inconsistent with a role of caveolin in activating this pathway.\textsuperscript{175} Equally confusing are the findings that caveolin-1-null mice show a somewhat similar phenotype with hyperactivation of ERK1/2 limited to areas of fibrosis,\textsuperscript{176} and that cardiospecific overexpression of caveolin-3 results in a phenotype with indications of dilated cardiomyopathy.\textsuperscript{177}

Strain-induced release of reactive oxygen species

Reactive oxygen species (ROS) may play an anabolic role in the myocardium, promoting growth and hypertrophy.\textsuperscript{178} This would be in keeping with the biology of the overloaded heart when ROS production is increased. Mechanical strain of myocytes increases formation of $\text{O}_2^{-}$, and superoxide dismutase/catalase-mimetics decrease strain-induced protein accumulation, though their ability to reduce strain-induced ANF mRNA expression is less clear-cut.\textsuperscript{179} Other interventions which increase ROS production (mild hypoxia,\textsuperscript{179,180} the positively-inotropic cardiac glycoside ouabain\textsuperscript{181}) also promote growth, and antioxidants attenuate some of the transcriptional changes associated with ouabain-induced hypertrophy (including ANF expression).\textsuperscript{181} Endogenous ROS production is stimulated by GPCR agonists in cardiac myocytes, and may be involved in the induction of hypertrophic changes by these agonists.\textsuperscript{182-184} A proportion of strain-induced $\text{O}_2^{-}$ production is sensitive to AT1- or ET\textsubscript{A}-receptor antagonists, suggesting that local release of these agonists may lead to ROS production.\textsuperscript{179} Equally, however, the induction of hypertrophy by mild hypoxia was attributed to ET-1 rather than ROS.\textsuperscript{185} The ability of ROS to cause receptor PTK phosphorylation,\textsuperscript{186} recognized to be generally anabolic, has been mentioned earlier.
The origins of ROS in myocytes are unclear, though, they may arise from incomplete reduction of O₂ by the electron transport chain or the activities of membrane-bound NAD(P)H oxidase, xanthine oxidase and cyclooxygenase. Interestingly, hearts of mice in which a gene encoding one of the subunits of membrane-bound NAP(P)H oxidase had been disrupted were unable to initiate hypertrophy following infusion of subpressor concentrations of ANGII. The overall conclusions are that ROS are trophic factors under suitable circumstances, and they may play a role in any activation Ras or Akt during mechanotransduction.

Does Strain-induced Growth Really Exist?

One major question with respect to mechanotransduction is whether strain-induced growth per se (as opposed to attachment- or agonist-dependent growth) involves ECM-based signaling systems. Experiments with cyclically-strained myocytes adhering to a fibronectin matrix have shown that, although there is a significant AT1/ET₄-receptor-dependent effect of strain on BNP expression, an AT1/ET₄-receptor-independent component remains. Thus, inhibition of strain-induced BNP expression by a combination of losartan and BQ123 (presumably at maximally-effective concentrations) was only ~60%. However, RGD peptides (which interfere with interaction between fibronectin and integrins) or soluble fibronectin caused a complete inhibition of strain-induced BNP expression, as did certain anti-integrin antibodies alone or, with increased efficacy, in combination. Both the strain dependent- and the strain-independent-components involve ERK1/2 and other MAPKs (the p38-MAPKs). However, experiments with RGD peptides, exogenous fibronectin or anti-integrin antibodies are not simple to interpret. It is presumably implicit that
strain-dependent growth must involve ECM attachment whatever the mechanism because ECM attachment is the intermediary between the myocyte and the elastic membrane substrate. If this is wholly or partially disrupted, then it would interfere with the transmission of the deforming force, but it not necessarily mean that there is active participation of integrin-based signaling in strain-induced growth.

References


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