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Changes in Cardiac Phenotype in Hypertrophy and Failure: From Receptor to Gene

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Abstract. **The terminally differentiated adult cardiac myocyte cannot undergo cellular division. Growth of the heart in response to chronic hemodynamic overload therefore occurs through hypertrophy of the myocytes. The adaptation of the myocyte during hypertrophy not only involves an increase in cell size but also results in a change in phenotype through modification of the pattern of gene expression. From in vitro studies, it can be learned that agonists like angiotensin-II, endothelin-1, cardiotrophin, basic fibroblast growth factor, insulin-like growth factor-I, or stimulation with the α_1-adrenergic agonist phenylephrine can induce hypertrophy. In vivo studies suggest that especially angiotensin-II and endothelin-1 play a prominent role in induction of hypertrophy during overload. These agonists couple to classical seven-transmembrane spanning domain (serpentine) receptors, signaling through activation of the phosphoinositide pathway. This leads to generation of 1,2-diacylglycerol and activation of protein kinase C. Surprisingly, however, these agonists were also shown to activate the mitogen-activated kinase (MAPK) pathway that is typically activated by (growth factor) receptors harboring (intrinsic) tyrosine kinase activity. Increased mechanical forces exerted on the heart during overload also induce hypertrophy, partly through autocrine and paracrine factors such as angiotensin-II and/or endothelin-1. However, direct stimulation of MAPK pathways by stretch might also be exerted through cross-talk with an activated integrin-focal adhesion kinase pathway. Activated MAPK partly translocates to the nucleus, where phosphorylation processes are initiated that lead to altered transcription factor activity. Some transcription factors involved in expression regulation in the hypertrophic myocyte have now been implied, and knowledge concerning genetic *cis-acting* elements that are involved is also increasing. However, the complexity and interplay of different (and possibly still unknown) signaling pathways do not yet warrant a complete picture regarding the mechanism of in vivo hypertrophy development during overload.**

Key Words. **Hypertrophy, heart failure, signal transduction, gene expression, phenotype**

*I*n response to chronically increased workload, a compensatory mechanism in the heart is activated that results in an increase of the mass of the ventricular tissue. Ventricular myocytes are terminally differentiated cells that cannot undergo cell division *at large*. Thus, the increase of ventricular muscle tissue under this condition is accomplished by hypertrophy, i.e., an increase in size of individual myocytes.

Enlargement of cardiomyocytes is accompanied by a change in gene expression that results in alteration of the cardiac phenotype during hypertrophy. Some of these changes can be seen as a positive adaptation, e.g., induction of atrial natriuretic factor (ANF) or (in rodents) upregulation of β-myosin heavy chain (β-MHC) expression. On the other hand, detrimental modification of gene expression also occurs. In this respect, downregulation of the expression of the genes encoding, e.g., the sarcoplasmic reticulum Ca^{2+}–ATPase (SERCA2 gene) and the β-adrenergic receptor (β-AR) is thought to be important for the inevitable physiological outcome of prolonged stimulation of the hypertrophic process, namely, heart failure [1].

Many agents have been implicated in the regulation of the growth of the heart, including hormones like angiotensin-II (AngII) and endothelin-1 (ET-1), α_1-adrenergic neurotransmitters (e.g., the α_1-agonist phenylephrine, PHE, reviewed in [2]), interleukin-like agents such as cardiotrophin-1 (CT-1) [3], several growth factors [2,4,5], and as yet unidentified factors that mediate a direct response to increased mechanical forces. Some of these factors have been shown to be involved in hypertrophy induction in vivo. However, interpretation of results from in vivo studies is hampered by the possible simultaneous involvement of different factors as well as different cell types such as cardiomyocytes, cardiac fibroblasts, and endothelial cells. Furthermore, detailed analysis of the complex and rapid activation of signaling pathways is virtually impossible in the intact heart due to the presence of different cell types. Therefore, the main evidence for the involvement of the factors mentioned above in induction of hypertrophy comes from in vitro models. The most commonly employed in vitro model system is that of short-term cultured neonatal rat cardiac myo-

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cytes. These cells respond to hypertrophic agonists by an increase in protein synthesis, cell size, organization of contractile proteins, and changes in gene expression that are reminiscent of in vivo hypertrophy. In cultured cardiac myocytes, it was furthermore shown that diverse signal transduction pathways are activated, through which an extracellular signal is transmitted from the receptor(s) to the nucleus via kinase cascades.

This article focuses on the way in which cardiac myocytes sense and respond to the extracellular and intracellular signals that ensue from enhanced demand for work. Questions that will be addressed in this respect are, e.g., which changes occur in cardiac phenotype during hypertrophy and failure, which agonists can be implicated in signaling, which signal transduction pathways are activated, and how this activation is transmitted to the genetic machinery that dictates cardiac phenotypic changes during hypertrophy/failure.

Hypertrophy and Heart Failure

Prolonged hemodynamic overload of the heart, e.g., by infarction, hypertension, or volume or pressure overload, ultimately leads to hypertrophy of the left ventricular wall. This hypertrophic process involves signal transduction (described below) that leads to rapid induction of transcription factors (immediate early genes). At a later stage, contractile (e.g., myosin light chain-2, skeletal α-actin, and in rodents β-MHC) as well as noncontractile protein genes (e.g., ANF) are induced, not only resulting in growth of the cardiomyocyte but also leading to changes in the contractile function of the cardiomyocyte [1,6]. These changes can be judged to be a positive adaptation, allowing the heart to maintain a normal cardiac output under the overload conditions. Thus, the initial outcome is *compensatory* growth of the heart by 1) increased myocyte cell-size, with a well-developed contractile apparatus, 2) reexpression of the potent vasodilator (fetal) ANF gene in the ventricular tissue that is beneficial during hemodynamic overload, and 3) improved economy of contraction by reexpression of the (fetal) β-MHC isoform (in rodents) that exhibits a lower velocity of ATP cycling and thus dissipates less ATP in the form of heat generation.

In contrast to the compensatory hypertrophic phase, heart failure ensues during a second decompensatory phase. Although the exact "point of no return" is still unknown, decompensation occurs when the hypertrophic adaptation of the heart to an increased demand for volume or pressure work does not lead to alleviation of the workload. In this case, the continuation of hypertrophic stimulation induces severe alterations in gene expression. These lead to diminished capacity of the heart to perform work and thus to supply all tissues with enough oxygen and nutrients to sustain (active) life [7]. The changes in gene expression that are initiated in the hypertrophic phase lead not only to altered architecture of the muscle (remodeling accompanied by fibrosis) but also to catecholamine desensitization (β-AR downregulation) and to deterioration of contractile properties. The latter phenomenon may partly be explained by changes in expression of Ca^{2+}-handling proteins, e.g., decreased expression of the SERCA2 gene resulting in decreased velocity of Ca^{2+} uptake in the sarcoplasmic reticulum (SR) (i.e., decreased velocity of relaxation) and also leading to lower SR filling, with Ca^{2+} giving rise to reduced contractility [8].

Changes in cardiac phenotype during hypertrophy and heart failure will be depicted in more detail below.

Cardiac Phenotype in Hypertrophy and Failure

Initial studies regarding gene-expression regulation during hypertrophy and failure were performed in experimental animal models. In the last years, however, more and more data are derived from human, mainly end-stage heart failure tissue, largely confirming observations in the experimental models. Changes in gene expression during hypertrophy and failure of contractile proteins, membrane proteins, enzymes involved in energy metabolism, proteins involved in signal transduction, and hormones are summarized in table 1 [9–26]. The plethora of phenotypic changes that occur during development of hypertrophy suggests that the functional outcome for heart function will be complex. Changes in gene expression that relate to Ca^{2+} handling are discussed below in more detail.

The activity of the SR Ca^{2+} pump is decreased during hypertrophy, leading to reduced velocity of relaxation. The increased expression of the Na^{+}–Ca^{2+} exchanger might be a compensatory mechanism to the reduced SR function to increase the velocity of Ca^{2+} clearance from the cytoplasm. On the other hand, the increased expression of the Na^{+}–Ca^{2+} exchanger suggests that inward Ca^{2+} transport (by reversed Na^{+}–Ca^{2+} exchange) is enhanced after excitation of the myocyte, suggesting that this is a compensatory adaptation to the decreased SR filling with Ca^{2+} that is brought about by decreased SR Ca^{2+}-pump activity. The observation that the expression of the SR (ryanodine-receptor) Ca^{2+} release channel expression is decreased during heart failure furthermore shows that the involvement of the SR in Ca^{2+} movement during contraction/relaxation is decreased at several functional levels.

The activity of the SR Ca^{2+} pump is governed by a regulator protein, phospholamban. This protein, in its unphosphorylated state, inhibits Ca^{2+}-pump activity by association with the SR Ca^{2+} pump. The downregulation of phospholamban expression during hypertrophy that is associated with decreased SR Ca^{2+}-pump expression might therefore be a compensatory mechanism. Furthermore, the inhibitory action of phospho-

Table 1. *Genomic expression in heart tissue during hypertrophy and failure*

Subcellular localization and functional protein	mRNA- and/or protein-expression	Hypertrophy or failure (species)
Contractile proteins		
Myosin heavy-chain	Isoform shift[a]	Hypertrophy (h&nh)
Myosin light-chain	↑	Hypertrophy (h&nh)
Actin	Isoform shift	Hypertrophy (h&nh)
Troponin T	Isoform shift	End-stage failure (h)
Membrane proteins		
L-type Ca^{2+} channel (SL)	Controversial (↓)	Failure (h)
Na^+/K^+ pump (SL)	↓ (Isoform specific)	Failure (h&nh)
Na^+/Ca^{2+} exchanger (SL)	↑	Failure (h)
Voltage-gated K^+ channel (SL)	↓ (Isoform-specific)	Hypertrophy (nh)
Ca^{2+} channel (SR)[b]	↓	End-stage failure (h)
Ca^{2+} pump (SR)	↓[c]	Hypertrophy (h&nh)
Phospholamban (SR)	↓	Hypertrophy (h&nh)
Energy metabolism		
Creatine kinase	↓[d]	Failure (h)
Signal transduction		
G_s protein	=	Failure (h)
G_i protein	↓	Failure (h)
Protein kinase C	↓(Isoform specific)	Failure (nh)
β-Adrenergic receptor	↓(Isoform specific)	Failure (h&nh)
AngII receptor	↑	Hypertrophy (h&nh)
Others		
Inducible nitric oxyde synthase	↑	Failure (h)
Transcription factor MEF-1	↑	Hypertrophy (nh)

[a]In human atrium only.
[b]Ryanodine-sensitive Ca^{2+}-channel.
[c]Some controversy exists with regard to the correlation between mRNA level (decreased) and protein level (decreased or unchanged) [24–26].
[d]Activity measurement.
Note: The column *Hypertrophy or failure (species)* denotes the earliest point in time at which changes in expression were studied. Species relates to human (h) heart tissue or nonhuman (nh, mainly rat) experimental models. Data were taken from [9], together with [10–23]. ↑ and ↓ denote increased and decreased expression, respectively.

lamban is overcome through phosphorylation by protein kinase A (PKA), coupled to the β-AR through adenylate cyclase. Therefore, downregulation of phospholamban under conditions where the β-adrenergic receptor is decreased and the inhibitory G_i protein (inhibition of adenylate cyclase) is increased might also serve a compensatory goal.

Taken together, severe impairment of Ca^{2+} handling in the hypertrophied/failing heart can be attributed to altered expression of Ca^{2+}-handling proteins, despite several compensatory changes that exist as well.

Factors Involved in Induction of Hypertrophy

Most studies concerning the ability of single agonists such as hormones, growth factors, and neurotransmitter analogues to induce cardiomyocyte hypertrophy have been performed in vitro using cultured ventricular myocytes. The criteria used in these studies to judge whether an agonist can induce hypertrophy in vitro are very diverse. These criteria range from true hypertrophy (increased cell size, protein content, and development of a well-ordered contractile apparatus) to separate processes that are thought to be indispensable for or to accompany development of hypertrophy, such as expression of immediate early genes (c-fos, c-myc, c-jun, and EGR-1), reexpression of ANF in ventricular cells, increased myosin light chain-2 (MLC-2) expression, or SERCA2 downregulation. However, we must be aware that development of a single hypertrophy-associated process in vitro might not be typical for the entire hypertrophy process seen in vivo. Thyroid hormone, for example, activates a form of hypertrophy that is accompanied by increased SERCA2 expression [27]. Furthermore, other factors may activate only a limited number of processes that accompany hypertrophy—e.g., ATP, which activates the immediate early gene expression program but does not increase protein synthesis [28]. Lastly, it is now becoming apparent that ANF expression is not always a good marker for the induction of hypertrophy [29]. This finding shows that we have to be cautious in the extrapolation of in vitro results to a possible in vivo hypertrophic capacity of agents under study.

Results from in vitro studies indicate that an astounding number of different factors can induce (as-

pects of) hypertrophy. These agonists span the diverse families of signal transduction pathways known at present. Hypertrophy can be induced by agonist activation of seven membrane-spanning-domain (serpentine) receptors that couple to phospholipase C activation, as was shown for AngII (AT_1 receptor) [30, 31], ET-1 (ET-A receptor) [32], PHE (α_{1A}-adrenergic receptor) [33], and thrombin [2]. On the other hand, several growth factor receptors were also shown to have some hypertrophic capacity in vitro. Among these are the receptors for insulin-like growth factor-I (IGF-I) and basic fibroblast growth factor (bFGF) [2,4] that signal through intrinsic receptor tyrosine kinase activity. A further kinase pathway that was implicated in the induction of hypertrophy involves cytokine-receptor signaling, in the case of the cardiac myocyte activated by cardiotrophin-1 (CT-1) [3].

All the stimuli mentioned above are of a neurohumoral nature. It is, however, well documented that stretching of cardiac myocytes also leads to hypertrophy. It was subsequently shown that this stretch-induced hypertrophy involves an autocrine and paracrine mechanism, namely, the release of AngII by cardiac myocytes [34]. However, induction of hypertrophy by stretch is not totally inhibited by blockade of AngII receptors [35], suggesting additional stimuli to play a role in this process. A recent study indeed shows that another hormone, ET-1, is secreted by stretched cardiomyocytes [36]. ET-1 receptor blockade partly inhibited the increase in protein synthesis brought about by stretch. ET-1 and AngII were furthermore shown to synergistically activate the raf/MAPK pathway (described below) [36] that mediates signal transduction leading to hypertrophy. This finding suggests that mechanical overload of the cardiac myocyte leads to multifactorial induction of hypertrophy.

Changes in membrane stress (e.g., those that occur during cyclic stretch of cardiomyocytes) are normally sensed by integrins that convey an activation signal to a focal adhesion kinase (FAK) [37]. Thus, even a fourth class of signaling molecules might activate hypertrophy. However, this pathway has only been sparsely studied in cardiac myocytes in a model of swelling-induced activation, and evidence for involvement in transmittal of mechanical forces in stretch-induced hypertrophy is not yet available.

As mentioned above, the in vitro model of cultured cardiomyocytes that is used in most of the studies with regard to induction of hypertrophy circumvents the possible interplay of different cell types through paracrine mechanisms. However, we must bear in mind that the in vivo induction of hypertrophy will almost certainly involve several factors that may even sequentially interact during hypertrophy/failure development. A good example in this context is the corelease of ATP with the adrenergic agonist norepinephrine from nerve endings. ATP alone, through P_2 purinergic receptors, can activate immediate early genes but does not induce hypertrophy. However, the normal hypertrophic response to α_1-adrenergic agonist is inhibited in the presence of ATP [38]. This finding suggests that identification of all factors involved in in vivo induction of hypertrophy will not be easy.

Nevertheless, some in vivo models of cardiac overload have already given insight into the identity of factors that play a prominent role in hypertrophy induction and heart failure. Experimental left ventricular (LV) infarction in rats by coronary artery ligation leads to hypertrophy of remaining viable tissue, decreased contractility, and in the long run to decreased survival due to heart failure. After infarction, myocardial ET-1 levels are increased. Blocking the ET-1 receptor after infarction decreases contractility, indicating that the ET-1 increase probably helps to maintain cardiac contractility [39]. Surprisingly, however, despite the fact that ET-1 receptor blockade decreased contractility, this treatment also greatly improved survival of rats with chronic heart failure [39]. Since the ET-1 antagonism was accompanied by prevention of ventricular remodeling (hypertrophy), this agonist seems to play a prominent role in development of hypertrophy and failure during infarction-induced cardiac overload. Furthermore, hypertrophy of the heart provoked by hemodynamic overload after aortic constriction also involves ET-1. In this model, again it was shown that an ET-1 receptor antagonist blocks development of cardiac hypertrophy [40]. However, ET-1 only seems to plays a role in the early phases of pressure-overload hypertrophy since the effect of ET-1 receptor antagonism wears off after one week.

Another factor that is now directly implicated in the development of hypertrophy in vivo is AngII. After LV myocardial infarction by ligation of the coronary artery in rats, blockade of the AngII AT-1 receptor largely prevented development of left ventricular hypertrophy [41]. Furthermore, in a model of spontaneously hypertensive rats, the constantly exerted pressure overload induces severe hypertrophy that gradually develops with age. Administration of an AT-1 blocker at an age when hypertrophy is already present not only prevented further LV wall thickening but even led to regression of hypertrophy [42]. Moreover, chronic AngII infusion in rats led to increased LV mass, even at an AngII dose where bloodpressure was not increased [43].

These results indeed suggest that the development of LV hypertrophy under overload conditions has a multifactorial origin.

Transmembrane and Cytoplasmic Signaling

Signal transduction in cardiac myocytes elicited by agonists that are involved in induction of hypertrophy has been a research topic for over a decade. Until a few years ago, however, the main topic was signaling by

seven-transmembrane spanning domain (serpentine) receptors coupling to G-proteins. Over the last years, another class of receptors has been implicated in hypertrophy induction, namely, (growth factor) receptors that harbor intrinsic tyrosine kinase activity. Even more recent is the finding that yet another class of receptors can be associated with hypertrophy, namely, that of the cytokine receptors that signal through receptor-associated tyrosine kinases.

The description given above concerning activation of three *separate* kinase pathways (serpentine-, receptor tyrosine kinase-, and cytokine-receptor coupled) is an intentional oversimplification. As will be described below, one of the most surprising discoveries of the last years was the cross-talk between different kinase pathways; serpentine- as well as cytokine-activated signal transduction pathways cross-talk with the MAPK pathway. This finding seems to make the MAPK signaling pathway the converging point to which signal transduction elicited by AngII, ET-1, PHE and bFGF, IGF-I, and CT-1 is routed, all inducing hypertrophy.

Below, we will describe the kinase pathways employed by these receptors in general, followed by a description of signaling by specific hypertrophic agonists. A general overview of the signal transduction pathways is also given in figure 1.

Signal transduction in general

Serpentine receptors and the phosphoinositide pathway. Several seven-transmembrane-domain-containing receptors have been detected in cardiac myocytes. These include the phosphoinositide cycle-coupled receptors for the hypertrophic agents AngII, ET-1, and PHE [2]. Associated with these receptors are heterotrimeric GTP-binding proteins, of which the Gα subunit can bind GDP or GTP. Upon occupation, the receptor catalyzes exchange of GDP for GTP on Gα. This exchange activates the Gα protein and causes its dissociation from Gβγ. Both the Gα–GTP complex and Gβγ [44] can now regulate effector molecules. Signaling by G-proteins is transient because of the intrinsic GTPase of the Gα subunit.

Two classes of G-proteins are activated: G_q which activates phospholipase C (PLC), and G_i, which inhibits adenylate cyclase [45]. G_i activation results in decreased cAMP levels and decreased PKA activity. This change may serve to relieve the inhibition of c-Raf

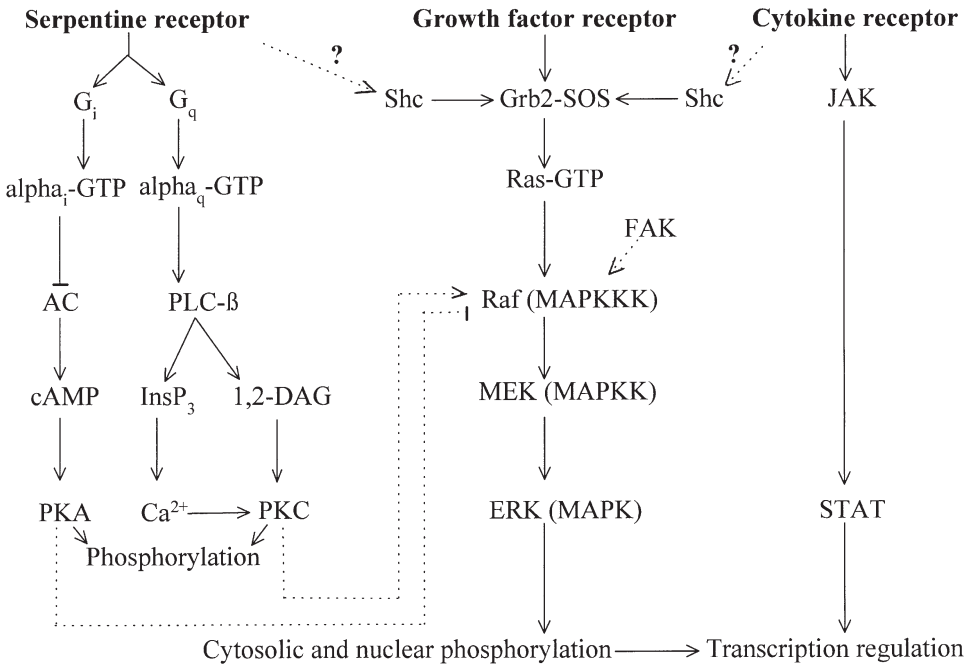

Fig. 1. *General overview of signal transduction pathways activated by serpentine- growth factor and cytokine receptors. Activation of serpentine receptors leads to inhibition of adenylate cyclase (AC) through the G_i GTP-binding protein, decreasing the activity of protein kinase A (PKA). This releases the inhibition that is brought about by raf phosphorylation. Through G_q, the same receptors activate phospholipase C (PLC), giving rise to protein kinase C (PKC) activation, leading to raf activation and MAPK activation. Growth factor receptors directly activate the MAPK pathway. Cytokine receptors activate transcription regulation through STAT and are thought to cross-talk to the MAPK pathway by a cytoplasmic adaptor protein Shc that might be involved in serpentine receptor signalling as well. Further details with regard to signal transduction and downstream events are detailed in the text. Arrows (↓) denote stimulatory pathways; ⊥ indicates an inhibition of the next enzyme; dashed arrows (⇢) denote cross-talk between the MAPK pathway and integrins (through FAK), serpentine-receptors, and cytokine receptors*

activation caused by phosphorylation by PKA, suggesting that this is a first point of cross-talk with the MAPK pathway, as will be described below. Activation of G_q is essential for PHE-induced hypertrophy and was shown to lead to activation of the PLC β-isozyme giving rise to production of inositol (1,4,5)-trisphosphate ($InsP_3$) and 1,2-DAG. In general, $InsP_3$ releases Ca^{2+} from intracellular stores. However, in cardiomyocytes this mechanism seems to be ablated by rapid phosphorylation and dephosphorylation of $InsP_3$ [46]. On the other hand, production of 1,2-DAG serves an important role in signaling since it activates protein kinase C.

The protein kinase C family of Ser/Thr kinases consists of at least 12 members that can be divided into three groups: classical cPKC isozymes, requiring Ca^{2+} and 1,2-DAG for activation; novel nPKCs, requiring only 1,2-DAG; and atypical aPKCs, for which the activation mechanism is still obscure. The presence of cPKC-α, nPKC-δ, nPKC-ε, and aPKC-ζ has been well documented in neonatal rat cardiac myocytes [45,47]. In contrast, only PKC-ε together with a low amount of PKC-δ is present in adult cardiac myocytes. The presence of PKC-β in cardiac myocytes is still controversial. Translocation of protein kinase C from the cytoplasm to a particulate (membrane) fraction is judged to reflect activation, as was the case for nPKC-δ and -ε after stimulation with ET-1 or PHE [45,47]. Direct involvement of PKC activation in the signal transduction pathway that leads to (aspects of) hypertrophy was shown in transfection studies employing constitutively activated PKC isoforms [48]. Downstream signaling from activated PKC towards gene regulation can either involve direct phosphorylation of nuclear proteins (histone, RNA- and DNA-polymerases) or cross-talk to the MAPK pathway through phosphorylation and activation of c-Raf (see below).

Tyrosine kinase receptors and the MAP kinase pathway. Growth factor protein tyrosine kinase receptors activate the MAPK, also called extracellular-signal regulated kinase (ERK) pathway (reviewed in [45,49]). These receptors homodimerize upon binding of the growth factor, whereupon autophosphorylation of an intracellular tyrosine residue occurs. This now serves as a docking site for adaptor proteins; the growth factor receptor-binding protein Grb-2 is bound to the receptor through its Src homology (SH2) domain, which is selective for phosphotyrosine-containing sequences. Another domain of Grb2, the SH3 domain, binds to SOS (son of sevenless). In this way, SOS, a guanine nucleotide exchange factor (GEF), is brought into the vicinity of membrane-bound ras, catalyzing the exchange of GDP for GTP on ras. Membrane-bound ras-GTP now binds to and activates raf (MAPKKK), the first enzyme of the MAP kinase pathway. Moreover, this raf-kinase is a substrate for PKC (stimulation) as well as for PKA (inhibition), indicating that this is the enzyme that translates multiple signals into one downstream phosphorylation event.

Several subfamilies of the MAP kinase pathways have now been unraveled, all signaling through a three-enzyme module. The (possibly physically joined) basic components are the following: 1) The MAPK kinase kinase MAPKKK (this is raf, as described above) is also referred to as MEKK (i.e., MAPK/ERK kinase kinase). This is a Ser/Thr protein kinase that becomes activated by interaction with ras-GTP and probably requires additional Tyr-phosphorylation for full activation. 2) MAPK kinase (MAPKK), the second component of the module, is also referred to as MEK (MAPK/ERK kinase). MAPKK is phosphorylated by MAPKKK on Ser-XXX-Ser/Thr, where phosphorylation of both sites is required for full activation. MAPKK itself is a so-called *dual specificity* kinase, since it is able to phosphorylate Ser/Thr as well as Tyr residues. 3) The third component of the module gives its name to the cascade: MAPK (or ERK) becomes phosphorylated by MAPKK on Thr-X-Tyr, again requiring dual phosphorylation for activation. MAPK itself is a so-called *proline-directed* Ser/Thr kinase that phosphorylates target proteins on a Ser/Thr-Pro motif.

In mammalian cells, the best-studied MAPK superfamily members, which are also present in cardiac myocytes, are ERK-1 ($p44^{MAPK}$) and ERK-2 ($p42^{MAPK}$), where the phosphorylation domain is Thr-Glu-Tyr [45,49]. These ERKs are activated by MEK1 and MEK2, which were detected in cardiac myocytes as well [5].

Several cytoplasmic substrates have been described for MAPK. The cytoplasmic protein PHAS-I (phosphorylated heat- and acid-stable protein) is a target for phosphorylation by MAPK, and complex formation between PHAS-I and eukaryotic initiation factor (eIF)-4E protein is decreased upon PHAS-I phosphorylation. In this way, mRNA Cap-binding protein eIF-4E becomes available to initiate complex formation between ribosomes and mRNA, stimulating protein synthesis. Another way in which the MAPK pathway can regulate protein synthesis is by phosphorylation and activation of $p70^{S6K}$ [50,51]. This S6 kinase, in its turn, phosphorylates the S6 protein in the 40S small ribosomal subunit, thereby stimulating protein synthesis. Phosphorylation of SOS, raf, and MEK by MAPK (ERK) point to a possible feedback regulation. Further feedback regulation is brought about by MAPK-induced phosphorylation of protein tyrosine phosphatase 2C (PP2C) [52], resulting in decreased protein phosphatase activity and prolongation of tyrosine kinase signal transduction. Here, another point of cross-talk between serpentine-and growth factor receptors exists; activation of PKC leads to phosphorylation and inactivation of protein tyrosine phosphatase PP2A [53], suggesting that tyrosine signaling can be prolonged by activation of PKC.

Upon activation, MAPK also partly translocates to the nucleus. There, several phosphorylation processes

can occur that mediate transcriptional regulation. One of the nuclear substrates for MAPK is $p62^{TCF}$ (ternary complex factor, or Elk-1). Upon phosphorylation, ternary complex formation between Elk-1, the serum response factor (SRF), and the serum response element (SRE) is stimulated, and in this way transcriptional activity from target genes is enhanced. This is also a diverging point for MAPK signaling; overexpression of $p44^{MAPK}$ (ERK1) but not of $p42^{MAPK}$ (ERK2) led to activation of Elk-1 [54]. Other transcription factors that are phosphorylated and activated are c-myc (by ERK2 and not by ERK1) and c-jun, together with RNA polymerase II. The data above indicate that activation of the MAPK pathway can stimulate protein and RNA synthesis in general but can also lead to the activation of specific genes, e.g., those containing the SRE.

Some observations directly implicate the MAPK (ERK) pathway in the development of hypertrophy. Microinjection of constitutively active ras in myocytes is accompanied by morphological changes reminiscent of hypertrophy and leads to increased ANF expression [55]. Furthermore, transfection with constitutively activated MEK increased the ANF promoter activity. In this study, cotransfection with wild-type (wt)-ERK2 led to enhanced stimulation of this promoter [56] as well as to activated β-MHC, skeletal α-actin, and c-fos promoter. Other data with respect to the involvement of the MAPK pathway in induction of hypertrophy by serpentine receptor agonists will be discussed below.

Another member of the MAPK superfamily that is present in cardiomyocytes is the stress-activated protein kinase (SAPK). Although this pathway is not activated by tyrosine kinase receptors, it might also be involved, through as yet unidentified cross-talk mechanisms with, e.g., the hypertrophy-inducing AngII pathway [57]. The SAP kinase, also known as JNK, i.e., c-jun NH_2-terminal kinase, activates c-jun through phosphorylation of amino-terminal serine residues. However, upstream activation signals for SAPK activation have only received limited attention in cardiac myocytes.

Cytokine receptors and the JAK/STAT pathway. Receptors for the cytokine family all share a β-receptor unit (gp130) in their signal transduction [58]. The specific receptor for the hypertrophic cytokine-like protein cardiotrophin-1 (CT-1) is the leukemia inhibitory factor (LIF) receptor. Upon dimerization with the cytokine-specific receptor complex (CT-1•LIF), gp130 transduces the cytokine signal to the cytoplasm. Although gp130 does not harbor intrinsic tyrosine kinase activity, dimerization leads to activation of gp130-associated tyrosine kinases. These Janus-associated kinases (JAKs), in their turn, directly phosphorylate and activate the dormant cytoplasmic transcription factors STAT (i.e., signal transducer and activator of transcription) that, upon translocation to the nucleus, can homodimerize or heterodimerize into sis-inducing factor (SIF) complexes that regulate sis-inducing element (SIE) containing target genes.

The signal transduction pathway initiated by cytokine receptors can, as was the case for serpentine receptors, cross-talk with the MAPK pathway. However, the mechanism of this cross-talk is largely unclear and is hypothesized to involve an adaptor protein that activates ras.

Agonist-specific signal transduction

Although it has been described that growth factor receptor-mediated signaling by IGF-I and bFGF induces hypertrophy, no cardiomyocyte-specific details concerning intracellular signal transduction are known. Therefore, only the best-studied pathways—that is, those activated by AngII, ET-1, and the α_1-adrenergic agonist PHE—will be described. Additionally, recent data about signaling through the LIF receptor that can be activated by CT-1 will be described.

Angiotensin-II. AngII, through the AT1 receptor and presumably through G_q, leads to activation of PLC in cardiac myocytes. This activation, however, is very transient, as reflected in low levels of inositol phosphate production [2]. On the other hand, the intracellular $[Ca^{2+}]$ was increased by AngII, suggesting that locally increased $InsP_3$ levels might release Ca^{2+} from intracellular stores [59,60]. Although an increase in 1,2-DAG by AngII was reported in cardiac myocytes [2], no data are available on PKC activation by AngII in these cells. However, AT1 receptor-transfected COS cells respond to AngII by increase in intracellular $[Ca^{2+}]$ as well as by activation of PKC-α and -ε [61].

Stimulation of cardiomyocytes by AngII also results in activation of the MAPK pathway; tyrosine kinase activity increased, and $p42^{MAPK}$ and $p44^{MAPK}$ were phosphorylated [60]. Downregulation of PKC by pretreatment with PMA did not suppress MAPK activation by AngII, suggesting that cross-talk between the AngII-activated phosphoinositide and MAPK pathways is not brought about by PKC. On the other hand, chelation of intracellular Ca^{2+} completely inhibited the activation of the MAPKs. The identity of the Ca^{2+}-regulated adaptor proteins that mediate cross-talk between the phosphoinositide and MAPK pathway is, however, still unclear.

Endothelin-1. In cardiomyocytes, ET-1 activates phospholipase C through G_q coupled to the ET_A receptor [32]. The $InsP_3$ level is not increased, which is also reflected in a near absence of increase in intracellular $[Ca^{2+}]$ [46]. In contrast, 1,2-DAG levels are increased, which results in the rapid translocation (activation) at low [ET-1] of PKC-ε, whereas PKC-δ is translocated at higher ET-1 concentrations [52]. Besides PLC, phospholipase D is activated, giving rise to 1,2-DAG production from phosphatidylcholine. It was shown that activation of PKC with phorbolester PMA (a 1,2-DAG

analogue) leads to PLD activation [62]. Therefore, PLD activation after ET-1 stimulation might be brought about by PKC and could represent a feedforward mechanism to prolong the supply of 1,2-DAG and PKC activity [52].

Another G-protein that is activated by ET-1 is G_i. As described above, this activation leads to a decreased PKA activity, possibly releasing β-adrenergic inhibition of c-raf kinase and leading to sensitization of the MAPK pathway.

In cardiac myocytes, stimulation with ET-1 also activated the MAPK pathway [36,52]. The stimulation is accompanied by raf (MAPKKK) activation that is at least partly dependent on activation of PKC. Activation of PKA leads to decreased raf activation by ET-1 [52]. Therefore, cross-talk exists between ET-1-induced raf activation (through G_q and G_i, as described above) and β-adrenergic inhibition of raf through phosphorylation by PKA. Activation of raf is reflected in activation MEK (MAPKK) and subsequently ERK1 and ERK2. This MAPK activation is detectable within five minutes and gradually declines to control values in an hour. The mechanism by which ET-1 activates the MAPK pathway may not only depend on the G_α subunits described above. ET-1 stimulation also leads to tyrosine phosphorylation of Shc (SH2-domain containing protein). It was described that phosphorylation of the abundant cytoplasmic adaptor protein Shc can be mediated through Gβγ subunits [63]. The Shc protein then might serve as an adaptor for Grb^2 by which SOS is activated, leading to ras activation as described for tyrosine receptors (see above), but this pathway has not been studied in cardiomyocytes.

Surprisingly, yet another MAPK superfamily is involved in signaling by ET-1. It was shown that stimulation of cardiac myocytes leads to activation of the stress-activated JNK/SAPK pathway that, among others, regulates c-jun activity [52]. However, no further details about the involvement of this cascade in hypertrophy induction are available.

α_1-adrenergic agonists. Activation of the α_1-adrenergic receptor by phenylephrine was reported to lead to activation of phospholipase C through G_q. $InsP_3$ levels are not increased due to rapid phosphorylation/dephosphorylation reactions, reflected in the absence of an increase in intracellular $[Ca^{2+}]$, as was the case for ET-1 [46]. This activation results in increased 1,2-DAG levels, and PKC-δ and PKC-ε are activated, judged by translocation to the particulate fraction [45].

Coordinated activation of the MAPK pathway is brought about by PHE, and this activation is partly sensitive to PKC depletion [45,64]. On the other hand, stimulation with PHE also resulted in a tyrosine kinase-dependent activation of ras. This finding suggests that crosstalk by PHE to the MAPK pathway is brought about by PKC as well as by tyrosine kinase-dependent signal transduction. The identity of the adaptor that mediates tyrosine-dependent ras activation might again be Shc, as described above for ET-1, but definite conclusions cannot yet be drawn.

Conflicting reports exist regarding the mechanism by which ras and MAPK are involved in ANF expression and morphological changes related to hypertrophy after α_1-adrenergic stimulation. By transfection of a dominant interfering ras mutant, it was shown that the α_1-adrenergic activation of MAPK and stimulation of the ANF promoter was dependent on ras activation [55]. In a second study, expression of dominant negative MAPKs interfered with activation of the promoter by constitutively activated ras but failed to block α_1-adrenergic stimulation of ANF promoter activity [65]. Furthermore, p24 and p44 MAPK activation by ATP or carbachol did not induce ANF expression, and inhibition of MAPKs did not block PHE-stimulated ANF promoter expression. This study thus suggests that ras-induced MAPK-dependent and PHE-regulated MAPK-independent pathways exist that regulate ANF expression. In a third study, depletion of p42 and p44 MAPK activity by antisense oligodeoxynucleotides was reported to inhibit the development of morphological features of hypertrophy as well as to attenuate the induction of ANF by PHE [66]. This implicates MAPK activity in all aspects of hypertrophy development. In a fourth study, expression of dominant negative MAPK led to inhibition of PHE-induced ANF reporter gene expression but did not prevent PHE-induced morphological changes [67]. Thus, definite conclusions with regard to the involvement of ras and/or MAPK in the regulation of ANF gene expression and development of hypertrophy await further study. On the other hand, a recent report indicates that, besides ras, a second small GTP-binding protein, rho, is involved in regulation of ANF expression after PHE stimulation [69]. Inhibition of rho attenuates α_1-adrenergic stimulation of ANF expression but does not interfere with ERK activation by PHE. This finding suggests that independent pathways exist that regulate ANF expression and MAPK activation after α_1-adrenergic stimulation.

Cardiotrophin-1. Direct intracellular signaling by CT-1 has not been described. However, it was reported that CT-1 binds to the LIF receptor. Therefore, until more data are presented on CT-1 signaling, we can take LIF-induced signaling to be representative for CT-1 as well. In cultured cardiac myocytes, LIF stimulated JAK phosphorylation within five minutes. The substrate for LIF, STAT, was also phosphorylated within five minutes and was gradually dephosphorylated by 60 minutes. On the other hand, both ERK1 and ERK2 were activated. The increase in MAPK activity was detectable within two minutes after LIF stimulation, was maximal at five minutes, and gradually decreased thereafter. These results show that the LIF/CT-1 receptor is coupled to JAK/STAT as well as MAPK activation. However, a direct link between these pathways

and hypertrophy induction by CT-1 has not yet been provided.

The data described above certainly implicate cross-talk between kinase cascades as an important aspect of G-protein (and possibly cytokine) signal transduction towards hypertrophy. At the same time, we must now realize that a complete picture of signal transduction has not been gained yet but certainly comprises diverse kinase pathways involving PKC, ras and rho, and ERK and JNK/SAPK kinases as well as JAK/STAT.

Gene Expression Regulation by Nuclear Signals

Regulation of gene transcription is brought about by activation or inhibition of transcription factors (*trans*-acting factors) that bind to DNA sequences (*cis*-acting elements) in the promoter of target genes. Studies into transcription from promoters of several cardiac genes, such as myosin light-chain-2, α- and β-MHC, and ANF genes, have revealed several *cis*-acting elements and *trans*-acting factors that are involved in cardiac myocyte-specific expression. However, relatively little attention has been given to the role of *cis*-acting elements in gene regulation by the MAPK pathway during hypertrophy.

Activation of the MAPK pathway seems to underlie the common mechanism by which hypertrophic stimuli transduce their signals to changes ion gene expression. In this section, two modes of hypertrophy-associated gene regulation by nuclear signals derived from activation of the MAPK pathway will be described, as well as multifactorial regulation.

SRE/CArG-mediated regulation

Upon activation, MAPK translocates to the nucleus, where several phosphorylation processes occur that mediate transcriptional regulation. Phosphorylation of the nuclear target Elk 1 enhances ternary complex formation between Elk-1, the serum response factor (SRF), and the serum response element (SRE). In this way, transcriptional activity from genes containing an SRE is enhanced. The *cis*-acting SRE is also known as CArG, and intact SRF is required for muscle-specific activation of promoters through this CArG box. This CArG element plays a role in muscle-specific expression of several contractile protein genes such as the skeletal/cardiac α-actin-, α-myosin heavy-, and myosin light-chain genes.

One of the earliest genes induced by hypertrophic stimuli is the c-fos gene. In a very elegant study, using in vivo gene transfer, the activity of the c-fos promoter coupled to a reporter gene was studied under conditions of ventricular pressure overload [70]. Wt-c-fos promoter (356 bp) activity was activated 3–fold by two-hour pressure overload. Deletion of the SRE from this promoter resulted in a loss of pressure-induced c-fos-reporter gene expression. The SRE alone was able to confer pressure responsiveness to the minimal promoter, indicating that this element is necessary and sufficient for pressure response. A point mutation in SRE that abolishes interaction with Elk-1 from the SRF abolished the pressure overload response. Furthermore, the SRE is also required (and sufficient) for the AngII-induced activation of the c-fos gene [31]. This funding suggests that MAPK (ERK1) activation followed by Elk-1 phosphorylation and enhanced SRF formation is the underlying mechanism for c-fos induction during (pressure-overload) induced hypertrophy.

TRE-mediated regulation

Besides upregulation of c-fos expression by MAPK activation as described above, other transcription factors are activated and/or induced during hypertrophy. It was shown that MAPK activation leads to phosphorylation and activation of c-jun. Moreover, the JNK/SAPK pathway (activated by, e.g., AngII and ET-1) also leads to activation of c-jun. From the simultaneous activation of c-fos and c-jun, we can predict that upregulation will occur of genes (promoters) that contain TPA responsive element (TRE) sequences. *Cis*-acting TRE elements are stimulated by the TRE-binding transcription factor complex AP-1 (activator protein-1) that consists of homodimers and heterodimers of fos and jun proteins. One of the genes that contains functional TRE elements is the ANF gene. It was reported that overexpression of c-jun in atrial cardiomyocytes in the presence of a human ANF reporter gene resulted in a dramatic increase in promoter activity that was dependent on the TRE element [71]. Surprisingly, overexpression of c-fos resulted in inhibition of the ANF promoter. However, the inhibitory activity of c-fos was located in the carboxy terminus of the protein, while conventional AP-1-dependent activity is located elsewhere in the molecule, suggesting dual regulation by c-fos through the AP-1 as well as other *cis*-acting elements. In a later study, it was in fact shown that a carboxy-terminal deletion mutant of c-fos could stimulate the human ANF promoter [72]. The stimulation of c-fos expression by MAPK (ERK-1) and c-jun activity by the MAPK as well as the JNK/SAPK pathways again depicts a cross-talk mechanism in the regulation of gene expression during hypertrophy where c-fos inhibits the stimulatory action of c-jun on ANF expression.

Another example of transcription regulation during hypertrophy is the ET-1 gene. This gene is activated by AngII in an AP-1- (TRE-) dependent manner under conditions where c-fos as well as c-jun expression is increased [57]. A minimal TRE-repeat promoter was shown in this study to confer the responsiveness to AngII as well.

Multifactorial regulation

Several studies indicate that regulation of gene expression is brought about by an interplay between sev-

eral genetic element/binding factors. One of the best-studied hypertrophic stimuli in this respect is the α_1-adrenergic agonist PHE. As described above, signal transduction proceeds through the MAPK pathway. Minimal promoter elements that could confer α_1-adrenergic hypertrophic upregulation were identified for the ANF, MLC-2, and skeletal α-actin promoters [73,74]. Besides other elements, all three promoters contained an SRE/CArG element, suggesting involvement of MAPK/Elk-1 signaling. Furthermore, ANF and MLC-2 contained a TRE (AP-1-binding element). This shows that upregulation of gene expression can involve SRE as well as the TREs described above.

The data described above indicate that activation of the Elk-1, c-fos, and c-jun by the MAPK is involved in some of the aspects of gene regulation during hypertrophy. However, more studies will have to be performed to delineate the full spectrum of transcription factors involved, such as c-myc, the early growth response gene EGR-1, and possibly unknown factors as well.

Future Directions

It is now evident that induction of hypertrophy is of multifactorial origin. Furthermore, a broad array of kinase cascades can be activated by each agonist, and crosstalk is more the rule than the exception. Although signal transduction and gene expression regulation have been the focus of research in past years, much remains to be learned.

At the moment, relatively little information is available concerning regulation of transcription factor activity that dictates the detrimental phenotypic changes during hypertrophy. Identification of the (regulation of) factors that govern expression of, e.g., SERCA2, phospholamban, and the β-adrenergic receptor during hypertrophy is now of utmost importance. This will ultimately enable us to devise strategies to intervene in the decompensatory changes that occur after prolonged hypertrophy, with the ultimate goal of prevention of heart failure.

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