Cell Calcium, Oncogenes, and Hypertrophy

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The cellular mechanisms of cardiac hypertrophy remain unclear despite tantalizing clues gleaned from a variety of experimental approaches. Here we examine the hypothesis that an increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) triggers the expression of proto-oncogenes, which in turn direct the characteristic increase in protein synthesis. New results from perfused ferret hearts are presented demonstrating that [Ca\(^{2+}\)]\(_{i}\) increases as a direct consequence of an elevation in perfusion pressure. It therefore seems plausible that [Ca\(^{2+}\)]\(_{i}\) constitutes the crucial link between the initial stimulus for hypertensive hypertrophy (elevated perfusion pressure) and the secondary alterations in gene expression. Nevertheless, further investigation will be required to establish whether changes in [Ca\(^{2+}\)]\(_{i}\) are necessary or sufficient to stimulate myocardial cell growth.

Cardiac myocytes can divide only in the fetal and neonatal periods; afterwards, the heart grows by an increase in the mass and volume of each individual cell (hypertrophy) not by an increase in cell number (hyperplasia). Although enlargement of cell size figures prominently in normal growth, hypertrophy also occurs pathologically as a reaction to hypertension. While it is now apparent that pressure overload suffices to induce protein synthesis and myocardial hypertrophy, the intermediate steps are still not entirely clear. This tutorial summarizes the evidence for and against oncogenes and cytoplasmic Ca\(^{2+}\) as vital links in this chain. An integrative hypothesis for the cellular mechanisms of hypertrophy is presented.

**Induction of Oncogenes by an Increase in Aortic Pressure**

Pressure overload induces myocardial hypertrophy by improving the efficiency of existing components of the protein synthetic pathway, as well as by expanding the maximal capacity of the cell for protein synthesis. This reaction is not dependent on an increase in myocardial work: when contractile pressure development is prevented by removing ventricular preload or by hyperkalemic arrest, protein synthesis still increases as perfusion pressure is raised. Therefore, the increase in aortic pressure itself plays an important role in the initiation of hypertrophy, but the biochemical signal linking this mechanical parameter to protein synthesis has still not been pinpointed.

Several proto-oncogenes constitute exciting candidate mediators linking elevated perfusion pressure to hypertrophy as gauged from models of hypertrophy induced by \(\alpha\)-adrenergic stimulation or by pressure overload. When oncogenes were first identified in neoplastic tissues, they were regarded as foreign DNA sequences that underlie neoplastic cell proliferation. They are now known to be related to a set of normal eukaryotic genes called proto-oncogenes, which differ from the transforming oncogenes by subtle structural mutations. The proteins encoded by known oncogenes and proto-oncogenes fall into a relatively limited number of functional groups: cytoplasmic protein kinases, membrane guanosine triphosphate (GTP) binding proteins, growth factors, growth factor receptors, and DNA binding proteins thought to modulate RNA transcription. Thus, the original concept of oncogenes has expanded well beyond the boundaries of neoplasia in light of the realization that they encode vital regulators of normal mitosis and cell growth.

At least three proto-oncogenes are expressed at measurable levels in pressure-overloaded hearts: c-myc, c-fos, and c-Ha-ras. DNA-binding proteins that influence cellular differentiation or proliferation are encoded by c-myc and c-fos, while c-Ha-ras codes for a GTP binding protein. Figure 1 shows the changes in the expression of proto-oncogenes that occur soon after acute aortic constriction. An increase in c-fos and c-myc messenger RNA (mRNA) can be detected as early as 30 minutes and 2 hours, respectively; the levels peak at 8 hours and return to baseline by 48 hours. In contrast to these two proto-oncogenes, which increase in expression rapidly but transiently, c-Ha-ras expression goes up only gradually, reaching a plateau by 48 hours (Figure 1).
The precise role of each of these proto-oncogenes in hypertrophy is not defined, but the time course of their induction certainly fits that which would be required to orchestrate the important early changes in contractile protein synthesis. The predominant myosin isoenzyme undergoes a transition within 24 hours after aortic constriction, whereas the accumulation of α-skeletal actin mRNA begins as early as 4 hours. The slower, sustained increase in Ha-ras gene expression can also be linked indirectly to hypertrophy, in that the ras gene products can affect phosphatidylinositol turnover. Activation of the inositol phosphate pathway figures prominently in the type of myocardial hypertrophy initiated by α₁-adrenergic stimulation.

What Biochemical Signal Induces the Increase in Proto-oncogenes?

Even if we accept the premise that proto-oncogenes are involved, the nature of the biochemical signal linking the increase in aortic pressure to their activation remains uncertain. Although direct evidence from myocardial tissue is lacking, results from other cell types implicate intracellular Ca²⁺. Cytoplasmic calcium ion concentration is closely regulated, despite the thermodynamic challenge of maintaining a 10,000-fold Ca²⁺ concentration gradient across the surface membrane [extracellular calcium concentration ([Ca²⁺]₀) is on the order of 10⁻³ M, whereas basal intracellular calcium concentration ([Ca²⁺]) approximates 10⁻⁷ M]. This tight regulation is necessary given the diverse roles of Ca²⁺ in intracellular signaling. From a cardiac perspective, the most prominent of these roles is in excitation–contraction coupling: a transient rise in [Ca²⁺], during each cardiac cycle initiates crossbridge cycling and force generation. In other biological systems, more sustained changes in [Ca²⁺], underlie adaptive processes such as long-term potentiation, the basic element of memory at the neuronal level. Proteins that are specialized to bind Ca²⁺ with high affinity, including troponin (for contraction) and calmodulin (for long-term potentiation), serve as the primary effectors.

Numerous lines of evidence reveal yet another important role for Ca²⁺, namely, as a mediator of trophic stimuli. In the pheochromocytoma-derived PC12 cell line, drugs that increase [Ca²⁺], including the calcium channel agonist Bay K 8644, induce c-fos expression. The induction is prevented by calcium antagonists or by incubation in a low extracellular Ca²⁺ concentration. Another way in which Ca²⁺ acts is by potentiating the effects of protein kinase C. In 3T3 cells and in thymocytes, both protein kinase C-activating and Ca²⁺-mobilizing agents are able to increase c-myc mRNA levels, and they do so in an additive manner, as shown in Figure 2. Each panel shows the effect of one given growth factor on [Ca²⁺], protein kinase C activity (as gauged by diacylglycerol formation or by protein phosphorylation), and c-myc mRNA. Note that the greatest induction of c-myc occurs with platelet-derived growth factor (Figure 2A), which increases both [Ca²⁺] and protein kinase C activity. The diacylglycerol analogues OAG (Figure 2C) and TPA (Figure 2D), which fail to increase [Ca²⁺], do not induce c-myc as intensely despite comparable activation of protein kinase C (as judged by the extent of 80 KDa protein phosphorylation). These results illustrate concerted effects of Ca²⁺ and protein kinase C on c-myc induction.

Several less direct observations also support the idea that Ca²⁺ features prominently in the initiation of hypertrophy. α₁-Adrenergic stimulation or angio-

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**Figure 1.** Line graphs showing time course of proto-oncogene expression, determined by soft-laser density scanning of RNA blot autoradiograms, in pressure-overload hypertrophy. Values as percentage of highest level of expression are plotted as function of time after acute aortic constriction. Reprinted with permission.

**Figure 2.** Plots showing changes in diacylglycerol formation (●), 80 KDa protein phosphorylation (○), c-myc messenger RNA (mRNA) levels (□), and cytoplasmic free Ca²⁺ concentrations (●) during exposure to platelet-derived growth factor (PDGF) (panel A), fibroblast growth factor (FGF) (panel B), and diacylglycerol analogues (OAG and TPA) (panels C and D). OAG, 1-oleoyl-2-acyl-glycerol; TPA, 12-O-tetradecanoylphorbol 13-acetate. Reprinted with permission.
tensin II are known to induce proto-oncogenes such as c-myc or c-fos followed by an increase in protein synthesis in heart or vascular smooth muscle cells. These interventions both activate phosphatidylinositol turnover to increase [Ca\(^{2+}\)], and protein kinase C activity. Stretch of myocardial cells increases [Ca\(^{2+}\)]\(_i\), accelerates phosphatidylinositol turnover, and stimulates hypertrophy. Taken together, these observations indeed suggest that an increase in [Ca\(^{2+}\)]\(_i\), with or without the cooperation of protein kinase C, can induce proto-oncogenes that encode DNA-binding proteins.

**Relation Between Aortic Pressure and [Ca\(^{2+}\)]\(_i\)**

A compelling picture would begin to emerge if we could demonstrate that [Ca\(^{2+}\)]\(_i\) is elevated as an immediate consequence of the increase in aortic pressure. Although such a response has been postulated, no direct evidence is available in its support. To obtain such evidence, it is necessary to quantify [Ca\(^{2+}\)]\(_i\) in intact hearts at several perfusion pressures. We measured [Ca\(^{2+}\)]\(_i\) throughout the cardiac cycle (i.e., the Ca\(^{2+}\) transient) in Langendorff-perfused, isovolumically contracting ferret hearts, as described previously. Gated \(^{19}\)F nuclear magnetic resonance (NMR) spectroscopy was used to detect signals from the fluorinated Ca\(^{2+}\) indicator 5F-BAPTA. Figure 3 shows the results of a typical experiment in which we measured Ca\(^{2+}\) transients at two different perfusion pressures. Figure 3A and 3B show fluorine NMR spectra from a heart loaded with 5F-BAPTA; each spectrum contains two peaks, one from the free ligand (~2 ppm) and another reporting the calcium-bound species (~8 ppm). From the areas under each peak, we can calculate the free calcium concentration by the formula

\[
[Ca^{2+}]_i = K_d \times \frac{\text{area under calcium-bound peak}}{\text{area under free peak}}
\]

where \(K_d\) is the dissociation constant of calcium-5F-BAPTA (approximately 300 nM at 30° C). The left column shows spectra obtained with the perfusion pressure set to 80 mm Hg, sampled at end-diastole (Figure 3A) and at peak systole (Figure 3B). After these spectra were obtained, we increased coronary perfusion pressure from 80 to 120 mm Hg (right column). Both diastolic and peak systolic [Ca\(^{2+}\)]\(_i\), increased, as is evident from the relative enhancement of the calcium-bound peak in the spectra. Figure 3C shows the entire Ca\(^{2+}\) transient at each of the two perfusion pressures. The amplitude of the Ca\(^{2+}\) transient increased quite dramatically when perfusion pressure was raised from 80 (Figure 3, left) to 120 mm Hg (Figure 3, right).

Results from five hearts subjected to the same protocol are summarized in Figure 4. Both diastolic and peak systolic [Ca\(^{2+}\)]\(_i\), increased in a statistically significant manner when perfusion pressure was raised from 80 to 120 mm Hg. The contractile pressure developed by the heart was also augmented in response to the increase in coronary pressure (see Figure 4).
Kitakaze and Marban\textsuperscript{41}). The exact mechanism by which \([Ca^{2+}]_i\) increases in response to perfusion pressure is not yet apparent. Calcium current,\textsuperscript{42-43} Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange\textsuperscript{44} and calcium release from the sarcoplasmic reticulum\textsuperscript{45,46} have been reported to be altered in hypertrophied myocardium, but it is not known whether such changes occur acutely in response to elevated perfusion pressure. Nevertheless, our results do make it clear that the increase in aortic pressure itself suffices to induce an increase in \([Ca^{2+}]_i\).

Figure 5A presents a unified working hypothesis for the initiation of myocardial hypertrophy by pressure overload and a number of other stimuli. Each of these initial trophic stimuli is now known to increase myocardial \([Ca^{2+}]_i\), (increase in aortic pressure, present study; myocardial stretch, Reference 34; \(\alpha\text{-}\)adrenergic stimulation, Reference 31; angiotensin II, References 32 and 33). \(\alpha\text{-}\)Adrenergic stimulation and angiotensin II also increase the activity of protein kinase C.\textsuperscript{31-33} The increases in \([Ca^{2+}]_i\), and protein kinase C additively induce proto-oncogenes such as c-fos and c-myc.\textsuperscript{22,24} These proto-oncogenes encode DNA binding proteins that modulate RNA transcription\textsuperscript{1,14,15} by binding to controlling elements of the transcribed genes.\textsuperscript{1,47} RNA translation itself also appears to be altered in several ways in hypertrophy, including increased ribosomal RNA assembly\textsuperscript{3} and increased efficiency of protein synthesis,\textsuperscript{2} although the mechanism is unclear. Recently, Klausner and Harford\textsuperscript{48} have reviewed one particular example of post-transcriptional gene regulation by changes in cytoplasmic metal ion concentration: an increase in intracellular iron inhibits the degradation of the transferrin receptor mRNAs. By analogy, it seems possible that the alterations of post-transcriptional gene regulation noted in hypertrophy might be induced by changes in intracellular Ca\textsuperscript{2+}.

A role for intracellular Na\textsuperscript{+} has been proposed,\textsuperscript{49} but even if the concentration of this ion rises, it may...
well act by secondarily altering \([\text{Ca}^{2+}]\), via \(\text{Na}^+\text{-Ca}^{2+}\) exchange.\(^{50}\) No cellular second messenger system is known to be dependent on \(\text{Na}^+\) per se, although such a possibility has not been excluded.

The exact mechanism whereby an increase in \([\text{Ca}^{2+}]\) might induce proto-oncogenes is still unclear. An increase in \([\text{Ca}^{2+}]\), can trigger the phosphorylation of a variety of cellular proteins by \(\text{Ca}^{2+}\)-activated, calmodulin-dependent protein kinase type II (CaM-kinase II)\(^{21}\) or by protein kinase C\(^{19,25}\). Although \([\text{Ca}^{2+}]\), and protein kinase C effects are known to be cooperative,\(^{24,25}\) other lines of evidence implicate CaM-kinase II as well. Morgan and Curran\(^{22}\) demonstrated that the induction of \(c-fos\) by an increase in \([\text{Ca}^{2+}]\), is blocked by the calmodulin antagonists chlorpromazine or trifluoperazine. They postulated a calmodulin-mediated or CaM-kinase II-mediated modification of a transcription activator protein that in turn would act to stimulate \(c-fos\) transcription. Thus, there is reason to believe that the effect of \(\text{Ca}^{2+}\) on proto-oncogene expression might be mediated by CaM-kinase II, although the data supporting this notion remain scanty.

**Unanswered Questions**

So far, we have made the case for this scheme as compelling as possible, but it is fair to review additional evidence for and against this hypothesis. Much, but not all, of the available data support the idea that an increase in cell \(\text{Ca}^{2+}\) induces protein synthesis. In favor of this notion, an abundance of extracellular \(\text{Ca}^{2+}\) allows hepatocytes to incorporate amino acids into new proteins fivefold to 10-fold more rapidly than in \(\text{Ca}^{2+}\)-depleted media\(^{52}\); the \(\text{Ca}^{2+}\) ionophore A23187, but not the \(\text{Na}^+\) ionophore monensin, can antagonize the inhibition of 3T3 cell protein synthesis by a bovine statoglycopetide\(^{53}\); and the calcium antagonist verapamil decreases the incorporation of \([\text{H}]\)proline into skeletal collagen and non-collagen protein.\(^{54}\)

Regarding striated muscle, there is more controversy. Kameyama and Ettlinger,\(^{55}\) using rat soleus muscle, have shown that A23187 increases total protein synthesis by 44% and that applied tension exerts an additive effect (see also Reference 56). On the other hand, Silver and Lewis and their respective colleagues\(^{57,58}\) failed to find a positive effect of A23187 or other calcium-mobilizing agents on protein synthesis. For myocardial tissue, Schreiber and coworkers\(^{59}\) have found a requirement for extracellular \(\text{Ca}^{2+}\) in the increase in protein synthesis during early pressure overload, but this phenomenon is not straightforwardly dose-dependent.\(^{59,60}\)

Second, it is still unclear whether activation of proto-oncogenes, including \(c-fos\) and \(c-myc\), is either sufficient or necessary for the initiation of hypertrophy. A cautionary note along these lines is sounded by the effects of \(c-myc\) in transgenic mice. When coupled to a cardiac-specific promoter, continual \(c-myc\) overexpression leads to myocardial hyperplasia, not hypertrophy.\(^{61}\) Differences in the time courses of various cellular responses also deserve further scrutiny. In response to stimuli that lead to hypertrophy, the increase of \(c-myc\) or \(c-fos\) expression is very rapid and short-lived.\(^{11,18}\) In contrast, the hypertrophic response to norepinephrine, for example, occurs much later (12-24 hours) and requires continuing exposure of cells to the catecholamine.\(^{27}\) Another example of a peculiar time course of gene expression is that of \(c-fos\), which exhibits a secondary peak at 8-12 hours\(^{11}\); the significance of this observation awaits clarification.

Third, it is important to note that the hypertrophic response to some stimuli does not require new proto-oncogene expression (see Figure 5B). In particular, the hypertrophy induced by thyroxine appears to be quite different from that induced by pressure overload.\(^{52,60}\) Triiodothyronine does not activate proto-oncogenes;\(^{1}\) instead, the hormone permeates the cell membrane and interacts directly with a nuclear protein (which, interestingly, is itself the homologue of the proto-oncogene \(c-erb-A\)) to modulate transcription.\(^{59}\) The end result differs in that the isozyme composition of the contractile protein (\(\alpha\)-myosin heavy chain) is quite distinct from that induced by pressure overload or by \(\alpha_1\)-adrenergic stimulation (\(\beta\)-myosin heavy chain, \(\alpha\)-skeletal actin).\(^{52,63}\)

Finally, it is worth noting the existence of another class of genes involved in tumorigenesis termed "tumor-suppressor" genes or "anti-oncogenes."\(^{65,66}\) In contrast to the oncogenes, the loss or inactivation of anti-oncogenes or their products is the key to transformation. One might speculate that cell growth is governed by separate, parallel growth-agonistic and growth-antagonistic signaling pathways, the balance of which determines whether a cell grows or remains quiescent. On the other hand, Whyte et al\(^{67}\) have reported a direct interaction of an adenovirus oncogene (E1A) product and that of an anti-oncogene (the retinoblastoma gene). Therefore, it is quite plausible that these two types of proteins are generally interwoven in a common signaling pathway for cell proliferation or growth. In principle, anti-oncogenes might play an important role in the regulation of hypertrophy, although it remains to be determined whether \(c-fos\) or \(c-myc\) proteins interact specifically with cellular anti-oncogene products.

In summary, further studies will be required to ascertain whether an increase in \([\text{Ca}^{2+}]\), is necessary or sufficient for the initiation of hypertrophy. Similarly, the precise role of proto-oncogenes remains to be clarified. As investigation proceeds at a brisk pace in this exciting area, the hypothesis presented here will surely require considerable refinement.

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