A “Wringing” Endorsement for Myosin Phosphorylation in the Heart

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Posttranslational modification of proteins influences a myriad of cellular processes including enzymatic pathways, membrane trafficking, and motility–contractility. In skeletal muscle, the phosphorylation of myosin modulates the calcium- (Ca$^{2+}$-) dependent regulation of contraction. Evidence provided by Davis et al. (1) of a marked gradient of both myosin light chain kinase (MLCK) expression and activity across the myocardium requires a reexamination of the physiological significance of cardiac protein phosphorylation in vivo.

The myosin II motor molecule of vertebrate striated muscle is a hexamer consisting of two heavy chains (HC, ~220-kDa) and two pairs of light chains (LC, ~20-kDa) (Figure 1). The HC s dimerize to form a double-headed, highly asymmetric molecule with head, neck, and tail domains. At the N terminus the HCs form two catalytically active globular heads, each of which contains an ATP- and actin-binding site. Linking the head and tail domains of each HC is the neck, which binds the essential (E-LC) and regulatory (R-LC) light chain subunits. Both LCs are important structural elements of the myosin motor; however, only the R-LC can be phosphorylated (Figure 1B) (2). Phosphorylation of the R-LC may induce structural alterations in the HC that alter the spatial relationship between the myosin catalytic domain and the thin filament. Electron microscopy of negatively-stained tarantula muscle suggests that phosphorylation of R-LC disorders the helical decoration of myosin heads on the thick filament (3). Phosphorylation of R-LC may thus “spring” the myosin heads from the thick filament, effectively reducing the inter-filament distance that the myosin cross-bridge must span in order to bind to actin and develop tension.

During the excitation–contraction coupling process, Ca$^{2+}$ released from the sarcoplasmic reticulum binds to the sarcomeric protein troponin C (TnC). This signal initiates a series of intermolecular movements within the contractile filaments that allows myosin to form a tension-generating complex with actin. Sarcomere shortening results from the ATP-hydrolysis-dependent cycle of cross-bridge attachment and detachment that converts chemical energy into mechanical work. In vertebrate striated muscle, phosphate incorporation by the R-LC modulates this Ca$^{2+}$ regulation of muscle contraction. For example, stimulation-induced increases in R-LC phosphorylation of mouse and rat fast-twitch muscle correlates with increases in the rate and extent of isometric twitch tension development—that is, a potentiated twitch contraction (4–7). Phosphorylation of R-LC is also associated with increased work output when mouse fast-muscle shortens against a load during a potentiated twitch (8). Thus, based on these studies, R-LC phosphorylation appears to augment the force, work, and power of submaximal contractions of mammalian striated muscle.

Data from the study of membrane-permeabilized muscle fibers have provided insight into the molecular basis for R-LC phosphorylation-induced modulations of muscle performance. In these fibers, R-LC phosphorylation increases the Ca$^{2+}$ sensitivity of the myofibrillar apparatus, thus decreasing the Ca$^{2+}$ concentration required to elicit a given, submaximal tension (9). This increased Ca$^{2+}$ sensitivity arises from a phosphorylation-induced increase in the rate at which cycling cross-bridges make the transition from non-force to force-generating states (10, 11). The mechanism for this effect may be that the addition of a negatively charged phosphate ion to the R-LC displaces the myosin head away from the negatively charged thick filament, thereby increasing myosin head mobility relative to actin and facilitating tension generation (12). Thus, R-LC phosphorylation-induced increases in the Ca$^{2+}$ sensitivity of tension development provides a molecular basis for the potentiation of twitch mechanics observed in intact skeletal muscle.

Although a functional role for R-LC phosphorylation in cardiac muscle contraction has yet to be fully established, new work by Davis et al. provides new insights (13). These authors used submaximally activated skinned fibers to show that, in addition to an increased Ca$^{2+}$ sensitivity, R-LC phosphorylation alters the stretch activation response—that is, the biphasic tension rise that occurs when a contracting muscle is lengthened. Phosphorylation of cardiac-like slow-twitch fibers decreased the difference between the tension minima that occur when the initial, rapid tension transient relaxes and the tension maxima that occur during the peak of the delayed, slower transient that follows. This dampened contractile response might result from the altered rate at which cycling cross-bridges are able to re-generate tension after perturbation by stretch (10, 11). Thus, R-LC phosphorylation mediated modulations of the stretch-activation response may have important implications for the heart, where oscillatory work is performed on a beat-by-beat basis.

In skeletal muscle, MLCK activity is regulated in a Ca$^{2+}$-calmodulin-dependent manner (6). Although cardiac R-LC can be phosphorylated (14–16), evidence for a cardiac isoform of MLCK is generally lacking. Instead, the heart seems to express both smooth and skeletal forms of MLCK (17). Important in this regard are the results by Davis et al. (1) that shed new light on MLCK expression and R-LC phosphorylation in the heart. These authors show that a skeletal-like MLCK is expressed in a spatially-specific manner across the myocardium, decreasing from the apex to the mid-ventricle region as well as across the heart wall, from the epicardium (large amounts of MLCK) to the endocardium (low amounts of MLCK). Evidence that this MLCK is active in the heart is provided by the pattern of R-LC phosphorylation, which corresponds to the pattern of MLCK expression—again, decreasing...
from apex to mid-ventricle and from epicardium to endocardium, respectively. This spatial pattern for R-LC phosphorylation may help explain, in part, why epicardial fiber shortening predominates over endocardial fiber shortening during ventricular systole, a force mismatch that produces the “wringing” motion that aids pump function. An R-LC phosphorylation-mediated increase in Ca$^{2+}$ sensitivity and decrease in the stretch-activation response of epicardial fibers, and the corresponding functional reversal of these parameters in endocardial fibers, may produce the force inequalities that lead to this functionally important torsional response. Given this possibility, future investigations regarding the functional significance for spatially specific distributions of MLCK and corresponding R-LC phosphorylation seem warranted. The prospect of targeting region-specific myosin phosphorylation in the heart may some day represent a new therapeutic avenue for tailoring cardiac hemodynamics in vivo.

References
Cytokines in Heart Failure: Potential Interactions with Angiotensin II and Leptin

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The hypothesis that the pathophysiology of heart failure involves, in part, an inflammatory process has been tested over the past decade (1, 2). Primarily, these studies have investigated the presence of proinflammatory cytokines and their receptors in the circulation or in cardiac tissue of patients with various etiologies and severity of heart failure. The most consistent finding is that plasma concentrations of tumor necrosis factor–α (TNF-α) and its circulating receptors are elevated and correlate with the severity of heart failure, as well as with poor prognosis of the patient (2, 3).

Cytokine expression in the myocardium is perhaps more compelling evidence of a proinflammatory process occurring during the progressive remodeling of heart failure. Evidence for the role of proinflammatory cytokines in the process of pathological left-ventricular remodeling comes from studies using transgenic models, as well as from recent research with implanted left-ventricular-assist devices (LVAD) in humans. Transgenic animals that overexpress TNF-α specifically in the myocardium develop fibrotic and dilated hearts with decreased function, and show a lift-fold increase in interleukin-1β (IL-1β) gene expression compared to that found in wild-type animals (4). These changes can be alleviated by infusing the transgenic mice with a TNF-α-specific monoclonal antibody, or by early (prior to onset of cardiac hypertrophy) treatment with batimastat, a matrix metalloproteinase inhibitor (5). Batimastat is thought to relieve the disease process by decreasing TNF-α-induced matrix protein synthesis, denaturation, and degradation, which contributes to left ventricular remodeling and dilation.

The remodeling of the ventricular wall to a more dilated state marks the progression to end-stage heart failure for the patient and, at the present time, does not appear to be reversible by conventional pharmacological therapies. Recent advances using LVAD provide relief to patients with rapidly decompensating heart failure and allow the left ventricle to begin the process of reverse remodeling. Two dramatic studies have supported the role of proinflammatory cytokines in this pathologic remodeling process and demonstrate the effect of LVAD in decreasing cardiac proinflammatory cytokine levels. Torre-Amione et al. observed that TNF-α is diffusely present in the left ventricle of patients at the time of LVAD implantation, but that the amount of TNF-α decreases nearly 95% in the left ventricle following the use of LVAD for 16–62 weeks (6). Birks et al. compared the production of TNF-α, IL-1β, and IL-6 in left ventricular samples from patients with decompensating heart failure who required LVAD support to those from patients with less severe heart failure undergoing elective heart transplantation (7). RNA transcripts of TNF-α and IL-6 were increased twofold and 2.5-fold, respectively, whereas the expression of IL-1β transcripts was increased tenfold in the LVAD patients compared to those observed in patients with New York Heart Association- (NYHA)-defined class III or IV heart failure.

The stimulus for the increased production and activity of cytokines is unknown, but it does not appear to be due to increased inflammatory cell activation (8). In addition to pressor and hypertrophic effects, recent...
findings suggest that the neurohormone angiotensin II (AII) may interact with or promote the expression of cytokines to effect the progression of heart failure (9). The angiotensin converting enzyme (ACE) inhibitor drug class is an essential tool in the treatment of chronic congestive heart failure. Patients treated with ACE inhibitors or all type 1 receptor (AT1R) antagonists have decreased concentrations of circulating TNF-α and some studies suggest that higher doses of ACE inhibitors will routinely decrease circulating levels of the proinflammatory cytokine IL-6 (10–13).

However, other enzymes in addition to ACE are capable of generating AII in the heart, and chymase, which is not blocked by ACE inhibitors, may allow for local generation of AII, decreasing ACE inhibitor efficacy (9). The intracellular pathways by which AII stimulates cytokine production involve protein kinase C (PKC) and, subsequently, the activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (1–14). It is interesting to note that TNF-α and IL-1β are potent inducers of all receptor expression (15, 16), perhaps setting the stage for a vicious cycle of AII-induced myocardial cytokine production and cytokine-induced increases in AII activity (Figure 1).

The initial discovery that TNF-α levels are elevated in heart failure was not driven by the notion of heart failure as an inflammatory process, but rather, that heart failure patients frequently present with a syndrome known as cardiac cachexia (chronic wasting of adipose tissue and skeletal muscle, while sparing the cardiac muscle). TNF-α (also termed cachectin) was hypothesized to mediate this syndrome (17), and indeed, the concentration of TNF-α is typically higher in the circulation of cachectic versus non-cachectic heart failure patients (18). TNF-α is thought to mediate muscle and adipose wasting by activating proteasomes and NF-κB–dependent pathways in skeletal muscle and adipocytes (19). The decrease in cardiac function (cardiac suppression) elicited by TNF-α is thought to be mediated through the activation of cytokine-induced nitric oxide synthase (iNOS), ceramide and sphingomyelin pathways, NF-κB activation, and the eventual uncoupling of β1-adrenergic signaling. Interestingly, IL-1β shares the cardioprotective pathways of iNOS and ceramide activation. In addition, IL-1β may also suppress cardiac function by increasing cyclooxygenase-2 (COX-2) and phospholipase A2 gene expression, and by phosphatidylglycerol inhibition of cytokine activation, which results in NF-κB activation (20, 21).

Subsequent to the discovery by Levine and colleagues of the role of TNF-α in cardiac cachexia (17), another cytokine, leptin, has been characterized as a major regulator of body mass and appetite (22). Leptin is primarily produced by adipocytes, whereas its receptors are expressed in a variety of tissues, including the heart (23). The long form of the leptin receptor is similar to gp130, a member of the IL-6 receptor (IL-6R) family, which also includes the receptors for leukemia inhibitory factor (LIF), and cardiotrophin I (24). The long form of the leptin receptor is unusual because it forms homodimers exclusively, whereas other members of the IL-6R family are capable of forming homodimers.
or heterodimers with other members of the gp130 class. Leptin receptors signal primarily by activating Janus kinase 2 (JAK2) and signal transducers and activators of transcription 3 (STAT3) in the majority of tissues studied, and also appear to activate suppressor of cytokine signaling 3 (SOCS3) (25). The potential for leptin to modulate the activity of other cytokines has been reported (26, 27) and this modulation may include interference with NF-κB effects by STAT3 (19). The ability of leptin to modulate NF-κB activity through the phosphorylation of STAT isoforms may also affect the ability of AⅡ to induce production of TNF-α and IL-1β, but this hypothesis has not been tested.

Leptin suppresses cardiac contractility through a nitric-oxide- (NO)-dependent pathway, and this cardiac suppression appears to be blunted in hypertensive animals (28, 29). However, these studies should be interpreted cautiously because the concentrations of leptin that achieved these effects were beyond that which is physiologically attainable. Nonetheless, physiologically relevant concentrations of leptin (25 ng/ml) are capable of completely abolishing IL-1β-induced decreases in cardiac contractility, suggesting a potential role of leptin in modulating proinflammatory cytokine effects in the heart during the progression of heart failure (30). Recent studies have examined circulating levels of leptin in various stages of heart failure and have compared concentrations in cachectic vs. non-cachectic patients (31–33). In general, these studies have found that leptin is increased in non-cachectic heart failure patients, but that cachectic patients have normal-to-low circulating levels of leptin. These findings are intriguing if, indeed, leptin is protective against the cardiodepressive effects of TNF-α or IL-1β. By facilitating the decrease of circulating concentrations of leptin, cardiac cachexia may facilitate the downward spiral of cardiac decompensation effected by elevated amounts of TNF-α or IL-1β.

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important genes, termed *Sle1a, Sle1b, Sle1c*, and *Sle1d*—result in a loss of tolerance to certain nuclear antigens (proteins). The functions of the products of these four genes are further modified by the participation of suppressor genes. Mutation of *Sle2* leads to the diffuse activation of B lymphocytes, and expression of certain alleles at the *Sle3* locus leads to a lymphoproliferative phenotype resulting from diminished apoptosis. These genes interact in an additive and epistatic fashion to produce features of SLE, such as the production of nuclear-protein–specific antibodies and inflammatory renal disease.

Fas is another important gene implicated in autoimmunity. In both humans with ALPS (autoimmune lymphoproliferative syndrome) (4) and in mice (5), loss-of-function mutations in the apoptosis-inducing receptor Fas or in its ligand (FasL) lead to dramatic accumulation of nonmalignant lymphocytes in peripheral lymphoid organs, accompanied by systemic autoimmunity. The recessive Fas mutation in mice is known as *lpr*, and the recessive FasL mutation as *gld*. Autoimmunity in individuals with lesions in the Fas apoptotic pathway is thought to result from impaired apoptotic deletion of autoreactive peripheral B and T cells, whereas coincident lymphoproliferation reflects the accumulation of these and of many additional lymphocytes, which would normally be deleted in the course of immune responses. It is especially striking that anergic (immunologically unresponsive) cells with unusual phenotypes progressively fill lymph nodes and spleen. Most of these cells, unlike normal mature T cells, express neither CD4 nor CD8. Though they express T-cell receptors and other T-cell markers, they also bear certain B-cell markers such as the CD45 isoform B220.

The degree and nature of autoimmunity caused by Fas mutations is highly dependent on ill-defined background genes. In C57BL/6 mice, for example, the *lpr* mutation causes mild autoimmunity, whereas MRL mice containing a mixed genetic background develop a fulminant lupus-like disease accompanied by vasculitis when they inherit the *Sle3* locus leads to a lymphoproliferative phenotype.

**Box 1. Regulation of the Lymphocyte Pool**

Both T and B cells undergo rigorous negative selection early in their development, so that most autoreactive lymphocytes are eliminated through apoptosis before reaching the mature lymphoid compartment. Further negative selection proceeds even among mature cells, assuring that self-reactive and potentially disease-causing lymphocytes are removed, together with senescent or superfluous cells. Simultaneously, positive selection for useful lymphocytes occurs in newly maturing cells, mainly in the thymus and liver, and among fully differentiated lymphocytes during immune responses.

**Figure 1. Regulating activated peripheral T and B cells by Sle1 and possibly other genes.** The size of this compartment is also controlled by activation-induced cell death (AICD) through Fas, wild-type Sle3, or other apoptosis-mediating receptors. Factors favoring the accumulation of cells (designated “+”) lead to the entry of additional cells into the lymphoid pool (left arrow). Those genes promoting reduction in cell numbers (designated “−”) result in a smaller pool (right arrow).

deficient C57BL/6/ *lpr* mice, causes massive lymphoid enlargement and a robust SLE phenotype. These mice develop high levels of nuclear-protein–specific antibodies and a proliferative glomerulonephritis very similar to that observed in SLE. Prominent among the expanded cell populations in these animals are B1 B cells, which are found in the peritoneal cavity and produce certain autoantibodies, and CD4+ T helper cells. These results raise interesting questions not only related to the genesis of SLE, but also to the role of apoptotic pathways in the homeostasis of the normal immune system.

How do these SLE-causing genes interact to cause the formation of autoantibodies? Although it remains unknown which genes within the *Sle1* locus promote lupus, one component at least (*Sle1b*) is expressed in B cells (8), and it is likely that other genes within this locus also encode molecules of fundamental importance in the regulation of lymphocyte activation. These aberrant B (probably with the help of autoreactive T) cells produce histone-specific antibodies; however, the resulting autoimmunity falls short of a full SLE syndrome. The present study (7) argues strongly that it is activation-induced cell death (AICD)—mediated through Fas—that limits both B and T cell activation, causing selective apoptosis of activated lymphocytes. Very likely this occurs after the activation of mature B and T cells in the peripheral immune system, because lymphocytes in *lpr* mice undergo normal, intrathymic negative-selection (Box 1). The data also emphasize that multiple apoptotic pathways are used for the elimination of autoreactive lymphocytes. Although the degree of functional overlap between Sle3 and Fas was not directly addressed, findings imply that certain lymphoid subsets may rely more heavily on one apoptotic pathway than on another.

These data emphasize the complexity inherent in the potential interaction of genes that regulate the threshold of B-cell activation (CD19, CD22, Lyn, and SHP-1, for example) (9, 10), and the variety of genes that might mediate apoptosis [Fas, CD27, and several
members of the tumor necrosis factor (TNF) receptor gene family. Stimulation of one or more clones of autoreactive T or B cells may be insufficient to trigger autoimmunity because of the safeguard of regulatory apoptotic mechanisms, such as negative selection. The failure of negative selection to delete even one autoreactive T or B cell clone may underlie a lifelong risk for autoimmunity should autoreactive stimuli (from viruses, drugs, or environmental agents) activate autoreactive lymphocytes. Such activation need not require an exogenous agent, but could instead come from stochastic events leading to dysregulation of B or T cells.

These genetic findings have implications for the regulation of lymphoproliferation as well as for the control of autoreactivity. Notably, the degree of lymphoid enlargement induced by the lpr mutation in the Fas gene is much greater when the disease-associated allele of Sle1 is also expressed. Although lpr-induced lymphoproliferation was known to be dependent on background genes, Sle1 is the first such locus to be defined. This finding supports the view that the accumulation of lymphocytes is driven by their state of activation, presumably driven by the presence of activating immunogen, and is dependent on genes that regulate the response to the antigen (Figure 1). This view is consistent with the reduction in T-cell lymphoproliferation in lpr mice with defective or absent B cells, which results in global impairment in antigen presentation. It is important to note that the antigenic stimulus driving the lymphoproliferation need not arise from environmental conditions, because lpr mice raised under germ-free and antigen-free conditions can also have lymphoid enlargement nearly equivalent to that observed in animals housed in conventional facilities (11).

Even within ALPS families, in which affected members share the same dominant negative Fas mutation, it has been puzzling that certain individuals have minimal lymphoproliferation while others have dramatic expansion of their peripheral lymphoid compartment. The epistatic interaction of Fas and Sle1 or similar genes could explain the variability of the ALPS phenotype. The data from mouse studies imply that the net result of the interactions of other gene products that affect lymphocyte activation ultimately results in the ALPS phenotype. Nonmalignant lymphoproliferation is also seen in Sjögren’s syndrome (12) and in certain cases of HIV infection (13), although the cellular composition of these conditions differs markedly. These and other lymphoproliferative disorders may also require two or more genetic “hits.”

SLE genetics is advancing at a rapid pace. In the near future, we should expect to see further clarification of the function of Sle1-encoded proteins and how they interfere with immune tolerance. It should also soon become clear how it is that these aberrant genes interact with apoptosis-controlling genes to result in severe autoimmune disease and lymphoproliferation. An increased understanding of these mechanisms may lead to better therapy both for lymphoproliferation and autoimmunity.

**References**

erratum: Localization of Receptor-Mediated Signal Transduction Pathways: The Inside Story


Jason M. Haugh

The labels for the x–axes in Figure 1 A–C were missing and have been labeled Time (min; see online version). Numbers in panels A and B of Box 1 were missing and have now been added. In Figure 3A, an arrow linking PLC to DAG has been added. In one instance, “Ligand” has been changed to “PIP2.” In Box 2, two illustrations were missing and have been added to panels A and B. In Figure 4, an arrow linking “Free internal rec. and ligand” to “Internal R–L complexes” has been added. We apologize to the author and our readers for the error.

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