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Oxidative Stress and Sarcomeric Proteins

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Review

This article is in a thematic series on **Posttranslational Modifications of Cardiac Proteins**, which includes the following articles:

Integration of Troponin I Phosphorylation with Cardiac Regulatory Networks [*Circ Res*. 2013;112:355–366]

Posttranslational Modification and Quality Control Cysteine Oxidative [*Circ Res*. 2013;112:367–381]

Posttranslational Modifications: Emerging Regulation in the Cardiovascular System [*Circ Res*. 2013;112:382–392]

Oxidative Stress And Sarcomeric Proteins

Posttranslational Modification of Sarcoplasmic Reticulum Ca^{2+} ATPase

Posttranslational Modifications of Cardiac Myosin Binding Protein C

Jeffrey Robbins, Editor

Oxidative Stress and Sarcomeric Proteins

Susan F. Steinberg

Abstract: **Oxidative stress accompanies a wide spectrum of clinically important cardiac disorders, including ischemia/reperfusion, diabetes mellitus, and hypertensive heart disease. Although reactive oxygen species (ROS) can activate signaling pathways that contribute to ischemic preconditioning and cardioprotection, high levels of ROS induce structural modifications of the sarcomere that impact on pump function and the pathogenesis of heart failure. However, the precise nature of the redox-dependent change in contractility is determined by the source/identity of the oxidant species, the level of oxidative stress, and the chemistry/position of oxidant-induced posttranslational modifications on individual proteins within the sarcomere. This review focuses on various ROS-induced posttranslational modifications of myofilament proteins (including direct oxidative modifications of myofilament proteins, myofilament protein phosphorylation by ROS-activated signaling enzymes, and myofilament protein cleavage by ROS-activated proteases) that have been implicated in the control of cardiac contractility. (*Circ Res*. 2013;112:393-405.)**

Key Words: contraction ■ oxidative stress ■ protease ■ protein kinase ■ sarcomere

The production of reactive oxygen species (ROS) increases in the context of various cardiac disorders. ROS-activated mechanisms that contribute to ischemic preconditioning are cardioprotective. However, high levels of ROS production that overwhelm cellular antioxidant defense systems generally produce deleterious changes in contractile performance and lead to adverse cardiac remodeling. Some cardiodepressive actions of ROS have been attributed to the activation of signaling pathways that influence the expression, phosphorylation, or function of calcium regulatory proteins (such as sarcoplasmic reticular Ca-ATPase 2a and ryanodine receptor 2), leading to changes in the magnitude or timing of the calcium transient and an inadequate calcium-induced contractile response.[1–3] However, oxidative stress-induced modifications of contractile proteins, that are not associated with changes in intracellular calcium homeostasis, may also contribute to contractile dysfunction and the evolution of heart failure. This article focuses on ROS-induced structural modifications of the sarcomere, due to direct oxidative modifications of myofilament proteins, myofilament protein phosphorylation by ROS-activated kinases, or myofilament protein cleavage by ROS-activated proteases, that interfere with the transduction of calcium-dependent contractile responses. Modifications that are not the obvious or direct target of ROS-regulated processes are beyond the scope of this article.

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Non-standard Abbreviations and Acronyms	
AKAP	A-kinase anchoring proteins
ASK-1	apoptosis signal-regulated kinase-1
CaMKII	Ca^{2+}-and calmodulin-dependent protein kinase II
cMyBP	cardiac myosin-binding protein
cTn	cardiac troponin
HNO	nitroxyl
MHC	myosin heavy chain
MLC	myosin light chain
MMP	matrix metalloproteinase
Mst1	mammalian sterile 20-like kinase 1
$ONOO^-$	peroxynitrite
PK	protein kinase
PTM	posttranslational modification
ROS	reactive oxygen species
Tm	tropomyosin

Oxidative Modifications of Sarcomeric Proteins

The contractile apparatus consists of a parallel array of interdigitating thick and thin filaments that form the molecular motor that powers cardiac contraction (Figure 1). The thin filament backbone comprises 2 helical strands of actin monomers, the elongated tropomyosin (Tm) molecule that associates end to end to form a continuous strand along the actin filament, and troponin complexes (consisting of the calcium-binding cardiac troponin (cTn) C subunit, the inhibitory cTnI subunit, and the Tm-binding cTnT subunit) positioned at every seventh actin monomer along the thin filament. The thick filament comprises 2 myosin heavy chain (MHC) molecules complexed with 2 molecules of myosin light chain (MLC)-1 (essential light chain) and 2 molecules of MLC-2 (regulatory light chain). The smaller light chain proteins are positioned at the myosin lever arm, between the rod portion of the molecule that forms the thick filament backbone and the head region that contains the actin- and nucleotide-binding sites.

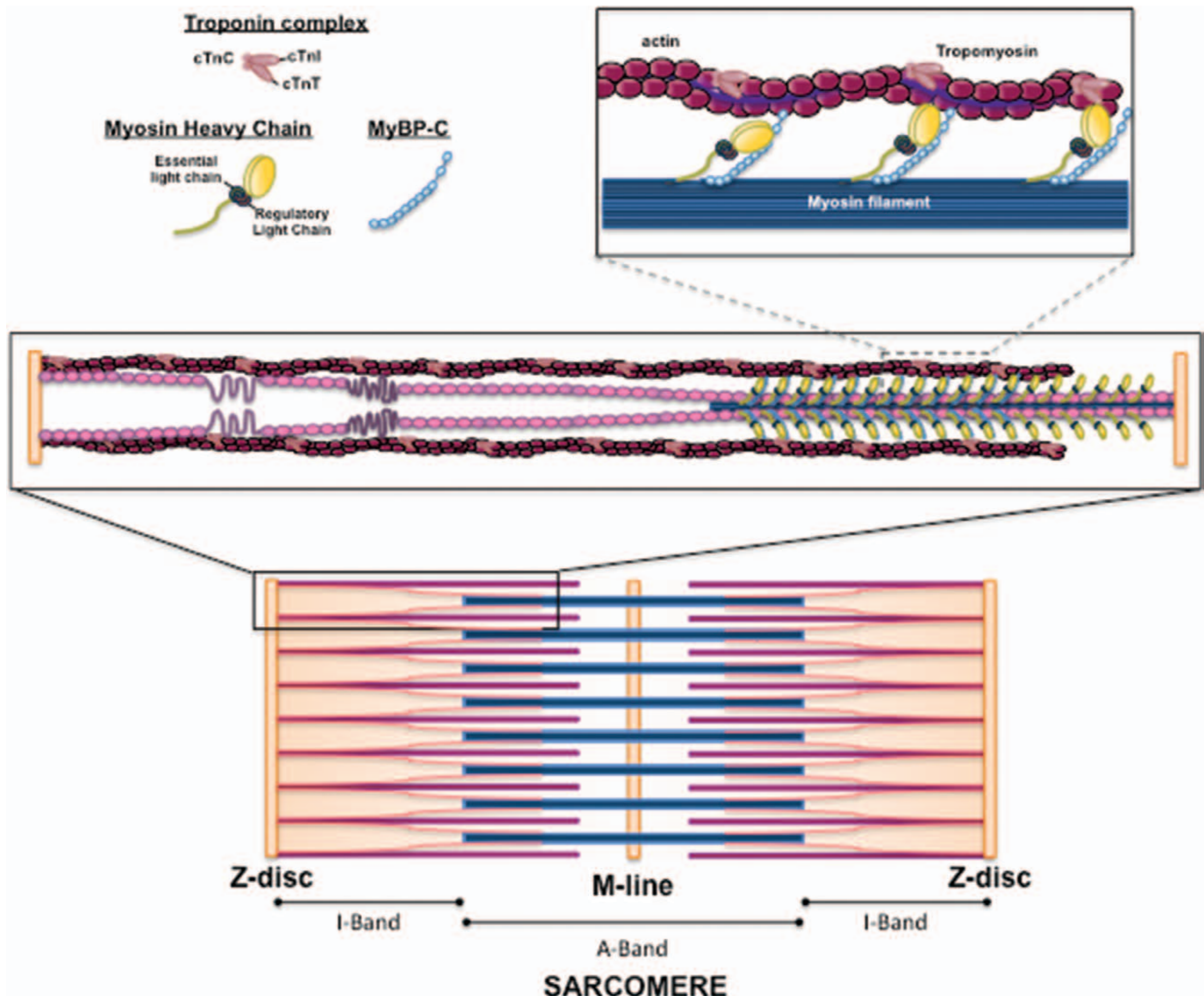

Figure 1. Schematic showing arrangement the major contractile and regulatory proteins in the sarcomere. cTnC indicates cardiac troponin C; and MyBP-C, myosin binding protein-C.

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Cardiac contraction is powered by cyclic interactions between the myosin motor and actin-containing thin filaments, with additional regulation provided by cardiac myosin-binding protein-C (cMyBP-C), a large multidomain thick filament protein located in the C-zone of the sarcomere. Titin, the third giant filament protein, runs from the Z disc to the M-band at the center of the sarcomere. Titin plays a role in the structural organization and assembly of myofibrillar proteins and functions as a molecular scaffold to recruit signaling molecules that influence mechanotransduction. Titin also contains 3 serially linked spring-like segments in an elastic I-band (the immunoglobulin-like domains, a proline, glutamate, valine, and lysine rich PEVK element, and an N2B element) that control the passive tension of the heart. The extensible elements in titin's I-band region are targets for molecular events (isoform splicing and posttranslational modifications [PTMs]) that fine-tune titin's elasticity.

Oxidative stress and increased formation of ROS (or reactive nitrogen species) can result in direct chemical oxidation (or nitrosylation) of many contractile proteins, leading to changes in their structural conformation and functional activity. Protein oxidation or nitrosylation generally occurs at reactive thiol moieties in cysteine (or to a lesser extent methionine) residues. The reactivity of any particular cysteine residue is determined by the pK_a of its thiol moiety; cysteine residues adjacent to basic amino acids, such as Arg or Lys, aromatic amino acids, or metal centers, have a relatively low pK_a (<6.5), are prone to deprotonate, and tend to be more susceptible to oxidation (Figure 2). The reaction between the cysteine thiolate anion and H_2O_2 results in the formation of sulfenic acid, a relatively unstable structure that typically reacts with other thiol groups to form intra- or intermolecular disulfide bonds. A reaction between the cysteine thiolate anion and NO (S-nitrosylation) or glutathione (S-glutathionylation) leads to the formation of mixed disulfides. This formation prevents further irreversible peroxidation of sulfenic acid to highly oxidized sulfinic or sulfonic acid species, that typically are more disruptive to protein structure and function. Other residues also can be targets for oxidative modification. When oxidative stress and superoxide formation is associated with increased formation of NO (eg, during early postischemic reperfusion or in the context of inflammation, where proinflammatory cytokines increase the expression of inducible NO synthase),[4,5] a near diffusion-limited reaction results in the formation of peroxynitrite ($ONOO^-$); $ONOO^-$ is a highly reactive compound that promotes protein tyrosine nitration, the addition of a nitro group (NO_2) to the 3 position of the tyrosine phenolic ring. $ONOO^-$ also can oxidize Cys residues and promote protein carbonylation, the addition of a carbonyl group to susceptible Lys, Arg, or Pro residues.

Cardiac contraction typically is reduced after treatment with oxidizing agents, such as superoxide anion or H_2O_2 (the more stable reactive species formed endogenously through spontaneous or superoxide dismutase-catalyzed dismutation of superoxide). Early studies in chemically skinned rat cardiac muscle fibers showed that superoxide anion depresses maximal calcium-activated force without changing calcium sensitivity or influencing rigor contracture in ATP-free solutions.[6] These results were interpreted as evidence that superoxide anion acts in a very specific manner to alter some aspect

Figure 2. Major redox modifications of cysteine and tyrosine side chains.

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of cross-bridge cycling (rather than some more nonspecific mechanism, eg, a proteolytic event that disrupts the structural integrity of the sarcomere). Initial attempts to expose mechanism showed that H_2O_2 treatment of isolated rat heart leads to the oxidation of thin filament proteins, both cysteinyl oxidation of Tm and cysteinyl oxidation/carbonylation of actin.[7,8] Oxidative modifications of Tm have also been detected, in association with the development of contractile dysfunction, in ischemic microembolized pig hearts and in the early postmyocardial infarction period in mouse hearts.[9,10] Oxidative modifications of actin (and protein kinase [PK] C-α) were detected during reperfusion of ischemic rat hearts.[11] There is evidence that oxidative modifications of cardiac Tm (at its single cysteine residue at position 190) leads to the formation of dimers that alter Tm's flexibility and interfere with Tm's interactions with other thin filament proteins. Although some investigators have argued that these structural events contribute to oxidative stress- and heart-failure-dependent changes in contractility,[12] this formulation ignores the many other ROS-dependent modifications of sarcomeric proteins that are detected in end stage human heart failure, that correlate with contractile dysfunction, and may also be contributory.[8]

$ONOO^-$ also decreases maximal force development of the intact heart and contractility in isolated human ventricular myocytes. Some of the cardiodepressant actions of $ONOO^-$ have been attributed to an increase in cGMP and the activation of a PKG-dependent pathway that decreases myofilament responsiveness to calcium.[13,14] However, $ONOO^-$ decreases force generation in isolated rat cardiac trabeculae in association with the nitration of MHC (and the myofibrillar isoform of creatine kinase, an additional target for a PTM that impairs contractility by disrupting myofibrillar energetic mechanisms).[15,16] In vitro studies performed on purified MHC show that $ONOO^-$ promotes myosin nitration, cysteine oxidation, and carbonylation at several highly reactive solvent-exposed sites in the catalytic subfragment-1 (S1) globular head region.[17] Functional studies suggest that these redox-induced PTMs (in particular, myosin oxidation at Cys^{707}/Cys^{697} and myosin carbonylation at Lys^{84}, which sits at a domain interface in close proximity to the reactive Cys^{707}/Cys^{697} residues) lead to a partial unfolding of the myosin subfragment-1, enhanced susceptibility to proteolytic cleavage by trypsin, and changes in Mg-ATPase activity (both increased intrinsic Mg^{2+}-ATPase activity and decreased actin-stimulated Mg^{2+}-ATPase activity).[15,17–19] However, there is reason to interpret the results of studies performed on purified myosin preparations in solution with caution, as some oxidative modifications of myosin (eg, Cys^{707} oxidation) are not detected in more physiologically relevant preparations (ie, in isolated cardiac myofibrils), where incorporation of myosin into the myofilament lattice leads to decreased cysteine reactivity.[20,21] In this regard, studies in an aging rat heart model identify myosin nitration at Tyr^{114}, Tyr^{116}, Tyr^{134}, and Tyr^{142} and pharmacological studies suggest that $ONOO^-$ decreases force generation by increasing myosin carbonylation. These studies conclude that nitration is not contributory and cysteine oxidation may actually be protective, as cysteine residues might act as $ONOO^-$ scavengers and prevent the $ONOO^-$-induced modifications elsewhere in the protein that disrupt functional activity).[17,22] The singular focus on myosin as the primary target of oxidative modifications may also be misguided, as ischemia/reperfusion injury leads to a decrease in maximum force per cross-sectional area and a decrease in rate of tension redevelopment in association with S-glutathionylation of actin in rat heart[23]; pro-oxidants such as glutathione+H_2O_2 or glutathione+diamide induce a high level of α-actin (not myosin) S-glutathionylation in isolated human cardiac myofibrils.[20] Actin S-glutathionylation at Cys^{374} (a site at the physiologically labile C-terminus) slows the kinetics of α-actin polymerization in vitro, destabilizes actin filaments in vivo, influences actin's role as a myosin-binding partner in the sarcomere, and decreases contractility; substitution of a glutathionylated form of actin for unmodified actin decreases maximal actomyosin-S1 ATPase activity.[24]

Oxidative modifications of other sarcomeric proteins have also been identified. $ONOO^-$ treatment or aging has been linked to increased nitration of tyrosine residues in a range of sarcomeric proteins, including cTnI, cTnT, MHC, MLC, Tm, cMyBP-C, actin desmin, and α-actinin.[22,25,26] Studies in human cardiomyocytes link α-actinin nitration to changes in cellular ultrastructure (disruption of the myofibrillar cross-striation pattern) and a defect in contractile function (reduced isometric force generation).[13] The less compliant titin N2B isoform has also been characterized as a redox sensor. Titin is coexpressed in the heart as N2BA and N2B isoforms that arise through alternative splicing of the transcript of a single gene. The principal difference between titin N2BA and N2B isoforms is in the length of their elastic I-band spring segment; N2B has a relatively short I-band segment and is very stiff, whereas N2BA has a longer I-band region and is more compliant. The shorter titin N2B isoform contains 6 cysteine residues that form ≥1 disulfide bonds under oxidizing conditions; disulfide bonding decreases the extensibility of N2B and leads to an increased cardiac stiffness.[27]

While oxidizing agents such as H_2O_2, superoxide, or $ONOO^-$ typically reduce force generation in skinned muscle preparations, nitroxyl (HNO, an electron reduction product of NO that displays very distinct chemistry and reactivity) acts in an antithetical fashion to increase force generation by increasing myofilament calcium sensitivity.[28] HNO reacts chiefly with cysteine thiols, forming either a N-hydroxlsulfenamide or (if there is a second cysteine in close proximity) inter- or intramolecular disulfide bonds (Figure 2). A recent study mapped HNO-dependent redox modifications in the sarcomere to strategically located cysteine thiols in actin, Tm, MHC, and MLC-1. The HNO-dependent formation of actin-Tm dimers (due to disulfide bridging between Cys^{257} in actin and Cys^{190} in Tm) is predicted to tether Tm to a position that is more permissive for Ca^{2+}-induced myofilament activation, thereby increasing contractility. The HNO-dependent formation of dimers between MHC and Cys^{81} in MLC-1 is predicted to enhance myofilament calcium sensitivity and would also improve cardiac contractility.[29] These recently identified redox-dependent modifications of myofilament proteins that enhance force generation represent promising targets for novel classes of inotropic agents that could be developed for the therapy of heart failure.

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Redox Regulation of Myofilament Protein Phosphorylation

Cardiac contraction must be dynamically regulated on a beat-to-beat basis to accommodate to changes in hemodynamic load and to respond to neurohumeral stresses. Much of this control is accomplished by signal-regulated PKs (or phosphatases) that regulate the phosphorylation state of strategically located Ser or Thr residues in various myofilament proteins (ie, myofibrillar protein phosphorylation is almost exclusively on Ser/Thr and not Tyr residues). Of note, many PKs that contribute to mechanical or neurohumoral control of cardiac contraction are also regulated by oxidative stress. This section focuses on phosphorylation events on the thin filament proteins cTnI and cTnT, the thick filament accessory protein cMyBP-C, and titin that are targets for redox-regulated enzymes.

Redox Regulation of Thin Filament Protein Phosphorylation

cTnI is the inhibitory component of the troponin complex that functions to fine-tune myofilament function to hemodynamic load; cTnI contains 3 well-described phosphorylation clusters at Ser^{23}/Ser^{24}, Ser^{43}/Ser^{45}, and Thr^{144}. cTnI phosphorylation at Ser^{23}/Ser^{24} (in the N-terminal region unique to cardiac TnI) is generally attributed to the β-adrenergic receptor pathway involving PKA.[30] cTnI-Ser^{23}/Ser^{24} phosphorylation accelerates the off-rate for calcium binding to cTnC, leading to a faster rate of cardiac relaxation (which is crucial to accommodate the β-adrenergic receptor-dependent positive chronotropic response). PKA is a heterotetramer enzyme consisting of 2 catalytic (C) subunits that are maintained in an inactive conformation by 2 cAMP-binding regulatory (R) subunits. cAMP activates PKA by binding to the R subunits; this interaction leads to the dissociation of the enzyme complex and frees the C subunit to phosphorylate target substrates. PKA holoenzymes are classified as type I or II based on the identity of the R subunit (RI or RII) in the enzyme complex. Cardiomyocytes coexpress both PKAI and PKAII enzymes that display distinct biochemical properties and subcellular localization patterns; PKAII is primarily recovered in the particulate cell fraction (in association with membrane scaffolding proteins, or A-kinase anchoring proteins [AKAPs]), whereas the type I PKA holoenzyme is recovered primarily as a cytosolic enzyme. Although RI and RII subunits share similar domain organization, there is genetic and biochemical evidence that RI and RII are not functionally nonredundant. In particular, PKAI functions as a redox-activated enzyme (Table 2). RI subunits contain a pair of redox-sensitive cysteine thiols within the N-terminal AKAP-binding region of the protein; these redox-sensitive cysteine thiols are not present in RII. The redox-sensitive cysteine thiols in RI form interprotein disulfide dimers that stabilize a conformation that binds AKAP proteins with higher affinity.[31] In cardiomyocytes, this is detected as a redox-dependent increase in PKAI binding to α-MHC, which has been characterized as a putative AKAP in the myofilament fraction.[32] In theory, RI dimerization might also control binding to cTnT, another myofilament protein recently identified as a sarcomeric AKAP,[33] but this has not been considered. Because the PKAI holoenzyme is activated by substrate-induced sensitization to cAMP (ie, it displays activity at low cAMP concentrations that do not support activation of type II PKA), the redox-dependent redistribution of PKAI to the sarcomere could allow for the phosphorylation of cTnI (and other sarcomeric substrates such as cMyBP-C, see below) and an increased cardiac contractility under conditions that are not associated with a β-adrenergic receptor-dependent increase in cAMP.[32]

The presence of distinct PKAI and PKAII activation mechanisms at the sarcomere allows for dynamic and nuanced control of myofilament function in response to various physiological and pathological stimuli. However, a redox-dependent mechanism that activates PKAI (via the RI subunit) may be counterbalanced by oxidative modifications involving a strategic located cysteine residue in the PKA catalytic subunit (at position 199 in the activation loop); S-glutathionylation at Cys^{199} (or the formation of internal disulfide between Cys^{199} and Cys^{343}) leads to a decrease in kinase activity.[34,35] Structural models suggest that the redox-dependent decrease in catalytic activity is because of a steric effect and reduced affinity for substrate,[34] but there is biochemical evidence that the cysteine thiol modification also decreases catalytic activity indirectly by facilitating the dephosphorylation of an adjacent threonine residue in the activation loop (a PTM that is required for kinase activity).[36]

Most studies have focused on cTnI-Ser^{23}/Ser^{24} phosphorylation as a PTM regulated by PKA, but this site also is a target for phosphorylation by other ROS-regulated Ser/Thr kinases. For example, autocrine/paracrine stimuli that activate the NO/cGMP pathway can promote cTnI-Ser^{23}/Ser^{24} phosphorylation by PKG.[37] PKG1α (a major PKG isoform in cardiomyocytes) contains a reactive cysteine at position 42 in the N-terminal homodimerization domain that abuts in the enzyme homodimer; oxidative stress leads to the formation of interprotein disulfide bonds that increase the enzyme's affinity for substrate and leads to a high level of cGMP-independent PKG1α catalytic activity. The N-terminus of PKG1β (the other major PKG splice variant in cardiomyocytes) does not contain a reactive cysteine at this position and is not activated by oxidative stress.[38] The redox-dependent mechanism for PKG1α activation seems to be important in the vasculature, where it provides for stimulus-specific mechanisms to control vasodilatation in response to NO and oxidative stress[39]; the functional consequences of a redox-dependent PKG1α activation mechanism in cardiomyocytes warrant further study.

Other redox-regulated signaling enzymes that can function as cTnI-Ser^{23}/Ser^{24} kinases include PKD, p90 ribosomal S6 kinase, and certain isoforms of PKC (Table 2).[40–44] PKD is a signal-regulated Ser/Thr kinase that phosphorylates sarcomeric proteins (cTnI, cMyBP-C) and regulates cardiac contractility; PKD also activates signal transduction pathways that regulate gene expression and contribute to cardiac hypertrophy.[45] The canonical pathway for PKD activation involves the growth factor receptor-dependent hydrolysis of membrane phosphoinositides leading to the formation of diacylglycerol and the colocalization of PKD with allosterically activated PKC isoforms at diacyglycerol-enriched membranes; this facilitates PKC-dependent transphosphorylation of PKD at Ser^{744}/Ser^{748} (2 highly conserved serine residues in the activation loop that regulate catalytic activity). The activated PKD enzyme then phosphorylates target substrates, typically

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Table 1. ROS-induced Modifications of Cardiac Sarcomeric Proteins

Protein	Phosphorylation	Reference	Functional Effect	Oxidation	Functional Effects	Reference
cTnI	Ser^{23}/Ser^{24} PKA, PKC, PKG, PKD, p90RSK	30,37,40–44	↑ Ca^{2+} dissociation from TnC ↓ Myofilament Ca^{2+} sensitivity ↑ Rate of relaxation	Tyr nitration		25,26
	Ser^{43}/Ser^{45}					
	Thr^{144} PKCβ Mst1 Y^{311}-phosphorylated PKCδ	 57,58 61 40	↑ Myofilament Ca^{2+} sensitivity Altered cTnI conformation and cTnI binding to cTnT/cTnC			
	Thr^{32}/Thr^{52}/Thr^{130} Mst1	61				
cTnT	Ser^{206} PKC Raf-1	 63 62	 ↓ Maximal force ↓ Myofilament Ca^{2+} sensitivity	Tyr nitration		25,26
	Thr^{197}/Ser^{201} PKC ASK-1	 63 69				
	Ser^{274}/Thr^{287} PKC	63				
Tm				Cys^{190} oxidation	↓ Contractile function Formation of Tm dimers ↓ Binding to actin ↓ Formation of actin-Tm complexes	7–10,12
				HNO-dependent Cys^{190} oxidation	Formation of dimers Cys^{257} in actin Tethers Tm to a position permissive for Ca^{2+}-induced myofilament activation	29
				Cys^{190} Carbonylation		8
				Tyr nitration	↓ contractile function	25,26
MHC				Cys^{697}/Cys^{707} oxidation	↓ Maximal force	17–19
				HNO-dependent Cys oxidation	Dimerization with Cys^{81} in MLC-1 ↑ Myofilament Ca^{2+} sensitivity ↑ Contractility	29
				Lys^{84} carbonylation		17
			Nitration Tyr^{114},Tyr^{116}, Tyr^{134},Tyr^{142}		↓ Contractile function	15,17,22
MLC-1				Tyr^{78}/Tyr^{190} nitration Cys^{81} nitrosylation	↑ Degradation by MMP-2 ↓ Contractility	25,99
MLC-2				Tyr^{152} nitration	↑ Degradation by MMP-2 ↓ Contractility	98
cMyBP-C	Ser^{282} PKA, PKC, CaMK, PKD	30,31 75	Primes MyBP-C for subsequent phosphorylation at Ser^{302} and Ser^{273}	Tyr nitration		25,26
	Ser^{302} PKA, PKC, PKD		Accelerates cross-bridge cycle kinetics			
	Ser^{273} PKA,PKC					
Actin				Cys^{374} oxidation	↓Tm-actin binding ↓ Maximal force ↓ Contractile function ↑ F-actin depolymerization ↓ Myosin ATPase activity ↓Actin filament sliding velocity	7,8,11
				Cys^{374} glutathionylation	↓ α-actin polymerization kinetics Destabilizes actin filaments Decreases contractility	20,23,24

(*Continued*)

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Table 1. (Continued)

Protein	Phosphorylation	Reference	Functional Effect	Oxidation	Functional Effects	Reference
				Cys257 oxidation by HNO	Formation of dimers Cys190 in Tm Tethers Tm to a position that is permissive for Ca^{2+}-induced myofilament activation ↑ Contractility	29
				Carbonylation	↓ Contractile function	8
				Tyr nitration	↓ Contractile function	25
α-actinin				Tyr nitration	Deterioration of cross-striated pattern ↓ Longitudinal force transmission	13,25,26
Titin	Ser469 PKA PKG	77,78,79	↓ Passive tension	Cys oxidation	S-S bond formation that decreases the extensibility of titin N2B ↑ Cardiac stiffness	27
	Ser11878/Ser12022 PKCα		↑ Passive tension			

CaMKII indicates Ca^{2+}- and calmodulin-dependent protein kinase II; cTn, cardiac troponin; cMyBP-C, cardiac myosin binding protein-C; MHC, myosin heavy chain; MLC, myosin light chain; MMP, matrix metalloproteinase; Mst1, mammalian sterile 20-like kinase 1; PK, protein kinase; ROS, reactive oxygen species; and Tm, tropomyosin.

at LxRxxpS consensus phosphorylation motifs.[46] In this regard, it is interesting to note that PKD displays a high level of in vitro cTnI-Ser23/Ser24 catalytic activity, although rodent (**P**v**R**rrS23S24) and human (**P**i**R**rrS23S24) cTnI sequences diverge somewhat from an optimal PKD consensus phosphorylation motif. However, there is ample evidence that PKD can phosphorylate substrates (such as c-Jun, β-catenin, and type IIα PI4P kinase) that do not conform to a LxRxxpS/T motif and there are hints in the literature that the flexibility of target substrate recognition may be enhanced during oxidative stress (due to the somewhat different ROS-dependent mechanism for PKD activation).[47–49] PKD is activated during oxidative stress via a mechanism involving c-Abl, which phosphorylates PKD at Tyr463 in its autoinhibitory pleckstrin homology domain.[50] This induces a conformational change that relieves autoinhibition and permits Src-dependent PKD phosphorylation at Tyr95.[51] Because the phospho-tyrosine at position 95 is a consensus-binding motif for the C2 domain of PKCδ, this leads to a docking interaction between PKD and PKCδ and PKCδ-dependent PKD phosphorylation at Ser744/Ser748. Stimulus-specific differences in PKD activation mechanisms (in response to growth factor receptors and during oxidative stress) have been linked to distinct functional responses in the vasculature; the prediction that the activation mode might also dictate the in vivo actions of PKD in cardiomyocytes has not been considered. Rather, studies to date show that endothelin-1 receptors recruit a PKD-dependent mechanism that promotes cTnI-Ser23/Ser24 phosphorylation, decreases myofilament Ca^{2+} sensitivity, and enhances contraction in adult cardiomyocytes,[42–44] but the endothelin-1 receptor-dependent increase in PKD1 activity does not couple to changes in cTnI-Ser23/Ser24 phosphorylation in cultured neonatal rat cardiomyocytes.[52] These divergent results suggest that stimulus-, age-, or disease-dependent differences in the cellular signaling machinery might influence PKD's signaling repertoire (and PKD-dependent control of contraction) in cardiomyocytes.

cTnI contains additional phosphorylation sites at Ser43/Ser45 and Thr144 that are traditionally viewed as a target for PKC.[53–56] Although the functional importance of Ser43/Ser45 phosphorylation remains uncertain, Thr144 is strategically positioned in the inhibitory region of cTnI where it can regulate calcium sensitivity and cross-bridge cycling rates. Thr144 phosphorylation has been attributed to PKCβ or the Tyr311-phosphorylated form of PKCδ (a form of PKCδ that accumulates during oxidative stress).[57,58] Of note, cTnI is phosphorylated by PKCδ in a stimulus-specific manner.[40] PKCδ phosphorylates cTnI exclusively at Ser23/Ser24 when it is allosterically activated by lipid cofactors. However, oxidative stress activates Src which phosphorylates PKCδ at Tyr311; the Tyr311-phosphorylated form of PKCδ displays a high level of Thr144 kinase activity—it executes coordinate cTnI phosphorylations at Ser23/Ser24 and Thr144.[40] This distinct cTnI phosphorylation pattern (ie, involving a dual phosphorylation at Ser23/Ser24 and Thr144) is functionally important, as cTnI-Thr144 phosphorylation alone has little effect on force generation or calcium sensitivity; Thr144 phosphorylation becomes functionally important in a Ser23/Ser24-phosphorylated background, where it prevents calcium desensitization because of cTnI-Ser23/Ser24 phosphorylation.[57] While these studies focus on a very specific ROS-dependent mechanism that fine tunes the enzymology of PKCδ, other redox modifications play a more general role to regulate PKC activity. For example, the lipid-binding C1 domain (ie, conserved module in the regulatory domain of all phorbol ester-sensitive PKCs) contains redox-sensitive cysteine residues; oxidative modifications at these sites lead to conformational changes that relieve autoinhibition and induce a high level of cofactor-independent catalytic activity. Redox modifications of the highly conserved cysteine residues in the catalytic domain activation loop have an opposite effect and disrupt PKC catalytic activity.[59]

Mammalian sterile 20-like kinase 1(Mst1) is a proapoptotic kinase that is activated via autophosphorylation and caspase-dependent cleavage of its autoinhibitory domain. Mst1 is activated in the context of ischemia/reperfusion injury and contributes to adverse cardiac remodeling.[60] Recent studies indicate that Mst1 interacts with and phosphorylates cTnI; phosphorylation has been mapped primarily to Thr32 (with some

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Table 2. Oxidative Modifications of Protein Kinases

Kinase	Posttranslational Modification	Functional Effects	Reference
PKA	RI subunit oxidation	↑ RI binding to AKAPs (α-MHC) ↑ PKAI kinase activity	32
	Catalytic domain Cys[199] -S-glutathionylation	↓ Kinase activity	34–36
PKG1α	Oxidation of Cys42 in the homodimerization domain	↑ Affinity for substrates ↑ cGMP-independent catalytic activity	38
PKC	Oxidation of C1 domain Cys residues	↓ Autoinhibition ↑ Kinase activity	59
	Calpain-dependent cleavage, release of a constitutively active catalytic domain fragment	↑ PKCα catalytic activity ↑ PKCδ-dependent phosphorylation of 14-3-3	68,85–87
	Oxidation of a conserved activation loop Cys	↓ Kinase activity	36,59
	Src-dependent phosphorylation of PKCδ at Tyr[311]	Altered substrate specificity, acquisition of cTnI-T[144] kinase activity	40
PKD	c-Abl- and Src-dependent phosphorylations of PKD at Tyr[463] and Tyr95 that relieve autoinhibition, promote PKCδ-dependent PKD phosphorylation at Ser[744]/Ser[748]	↑ Kinase activity	50,51
CaMKII	Met[281]/Met[282] oxidation	↑ Ca^{2+}-independent catalytic activity	76
ASK-1	Mechanisms that disrupt a C-terminal interaction with 14-3-3: Dephosphorylation of Ser[967] at the ASK-1 C-terminus or phosphorylation of 14-3-3 by ROS-regulated kinases (PKD, Mst1, catalytic fragment of PKCδ).Mechanisms that disrupt an N-terminal interaction with Trx-1 (Trx-1 oxidation)	↑ Kinase activity	66–68
Mst1	Caspase-dependent cleavage of an autoinhibitory domain	↑ Kinase activity	60

AKAP indicates a-kinase anchoring proteins; ASK-1, apoptosis signal-regulated kinase-1; CaMKII, $Ca^{2+/-}$ and calmodulin-dependent protein kinase II; cTnC, cardiac troponin C; MHC, myosin heavy chain; Mst1, mammalian sterile 20-like kinase 1; PK, protein kinase; ROS, reactive oxygen species; and Trx-1, thioredoxin-1.

additional phosphorylation at Thr[52], Thr[130], and Thr[144]). Mst1 also phosphorylates cTnT, but only when it is incorporated into the troponin complex; Mst1 does not phosphorylate free cTnT. There is evidence that the Mst1-dependent phosphorylation induces a conformational change in cTnI that alters its binding affinity for cTnT and cTnC.[61]

cTnT is another thin filament protein that contains phosphorylation clusters at Thr[197]/Ser[201]/Thr[206] and Ser[278]/Thr[287]—although studies to date suggest that T[206] is the only phosphorylation site that directly influences contractility. cTnT-Thr[206] phosphorylation has been attributed to PKC or Raf-1 (but not PKA or PKG) and is implicated as a mechanism that desensitized the myofilament response to calcium, decreases actomyosin Mg-ATPase, and depressed cardiac contractility.[62,63] cTnT also is phosphorylated by apoptosis signal-regulated kinase-1 (ASK-1), a ROS-regulated stress-activated mitogen-activated PK kinase that is abundant in cardiomyocytes.[64,65] ASK-1 contains a central kinase domain flanked by regulatory domains that engage in intermolecular interactions that limit ASK-1 catalytic activity. The interaction between the Ser[967]-phosphorylated C-terminal regulatory domain of ASK-1 and 14-3-3 proteins, and the interaction between the ASK-1 N-terminal regulatory domain and reduced thioredoxin-1, clamps ASK-1 in a configuration that maintains low basal catalytic activity. ASK-1 is activated during oxidative stress as a result of molecular events that disrupt these intermolecular interactions, including the ROS-dependent increase in the activity of cellular phosphatases that dephosphorylate the ASK-1 C-terminal Ser[967] residue, 14-3-3 protein phosphorylation by ROS-regulated kinases (such as PKD, Mst family kinases, or the catalytic fragment of PKCδ),[66–68] or thioredoxin-1 oxidation. ASK-1 activation has been linked to the activation of JNK or nuclear factor-κB pathways that influence apoptotic/necrotic cell death and adverse cardiac remodeling.[64,65] However, the activated form of ASK-1 also is detected in the sarcomere, where it phosphorylates cTnT.[69] ASK-1 activation leads to decreased cardiomyocyte contractility, but the link between cTnT phosphorylation and the cardiodepressant actions of ASK-1 remain uncertain, both because ASK-1 phosphorylates cTnT at Thr[197] and Ser[201] not Thr[206] (the site that has been implicated in the control of thin filament function)[63] and the activated form of ASK-1 also decreases the amplitude of Ca^{2+} transient (providing an alternate mechanism to explain the decrease in cardiomyocyte contractility).[69]

Redox Regulation of Thick Filament Protein Phosphorylation

cMyBP-C is a thick filament protein that is required for sarcomeric integrity, the regulation of cardiac contraction, and cardioprotection. cMyBP-C contains multiple phosphorylation sites in a linker region located between the Ig-like C1 and C2 domains in the N-terminal myosin-binding region of the protein (a region unique to the cardiac isoform of MyBP-C). An interaction between this region of cMyBP-C and the myosin subfragment 2 (S2) domain (a region close to the lever arm) influences thick filament packing and the kinetics of cross-bridge cycling; phosphorylation disrupts this interaction and accelerates cross-bridge kinetics. The 3 best-characterized cMyBP-C phosphorylation sites in this region are at RRTSer[273], RR(I/T)Ser[282], and LKKRDSer[302]; these 3 serine residues are flanked by sequences that support phosphorylation by basophilic kinases (and Ser[302] resides in an optimal

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phosphorylation motif for PKD).[70] Early studies established that all 3 sites are phosphorylated by PKA and that PKA also targets an additional in vitro phosphorylation site at Ser^{307}.[71,72] However, current literature suggests that phosphorylation is regulated in a hierarchical manner and that individual sites on cMyBP-C are differentially phosphorylated by PKC, PKD, p90 ribosomal S6 kinase, and Ca^{2+}, and calmodulin-dependent PKII (CaMKII). In particular, Ser^{282} is a phosphoacceptor site for PKA, CaMKII, or p90 ribosomal S6 kinase; phosphorylation at this site primes cMyBP-C for subsequent PKA-, PKC-, CaMKII-, or PKD-dependent phosphorylation at Ser^{302} (and PKA- or PKC-dependent phosphorylation at Ser^{273}).[71,73] Mutagenesis studies suggest cMyBP-C phosphorylation at Ser^{282} is sufficient to accelerate cross-bridge cycle kinetics (ie, that under certain circumstances this PTM can result in functional changes even without an increase in cMyBP-C phosphorylation at Ser^{302} or cTnI phosphorylation at Ser^{22}/Ser^{23}). However, there is also evidence that the physiological control of cardiac contractile function requires reversible phosphorylations at all 3 sites[73,74] and that electric field stimulation leads to an increase in Ca^{2+}-activated contractility at least in part through a mechanism involving PKD-dependent cMyBP-C phosphorylation at Ser^{302}.[75]

The ROS-dependent mechanisms that activate PKA, PKC, and PKD, that might underlie a redox-dependent increase in cMyBP-C phosphorylation, were considered in previous sections. CaMKII has been characterized as a ROS-activated PK (Table 2). CaMKII functions as dodecameric enzyme that comprises individual monomers containing 3 key structural elements: an association domain that controls assembly of the holoenzyme, a kinase domain that phosphorylates target substrates, and a regulatory domain containing an autoinhibitory motif that regulates catalytic activity. Stimuli that increase intracellular calcium and promote Ca^{2+}/CaM binding to CaMKII induce a conformational change that relieves autoinhibition. With prolonged increases in intracellular calcium, CaMKII executes an intersubunit phosphorylation at Thr^{287} in the autoinhibitory domain that prevents reassociation of the regulatory and catalytic domains and confers Ca^{2+}-independent catalytic activity. Recent studies indicate that the methionine residues at positions 281/282 in CaMKII's autoinhibitory domain (adjacent to the Thr^{287} phosphorylation site) are targets for oxidative modifications.[76] Oxidation at these sites leads to a high level of Ca^{2+}/CaM-independent CaMKII activity. Because oxidized and autophosphorylated forms of CaMKII share many cellular actions, a role for the redox-activated form of CaMKII as a cMyBP-C-Ser^{282} kinase is plausible and warrants future consideration.

Redox Regulation of Titin Phosphorylation

Changes in titin isoform expression during development and in disease provide a mechanism to regulate cardiac stiffness on a relatively long time scale. The relatively high elastic recoil of the perinatal heart is attributable to a low N2BA/N2B ratio, whereas an increase in the N2BA/N2B ratio in chronic heart failure leads to a decrease in passive tension. The spring-like segments in titin's elastic I-band also are targets for phosphorylation events that lead to more dynamic changes in cardiac elasticity. The serially linked spring-like segments in titin's I-band are differentially phosphorylated by PKA/PKG and PKC. PKA and PKG both phosphorylate a single serine residue at position 469 in the N2B segment, leading to a decrease in passive tension.[77,78] Because this residue is conserved in human cardiac N2BA and N2B isoforms, this PTM constitutes a general mechanism to regulate cardiac stiffness. PKCα phosphorylates cardiac and skeletal muscle titin isoforms primarily at different serine residues (Ser^{11878} and Ser^{12022}) in the PEVK domain; phosphorylation in the PEVK domain has an antithetical effect to increase passive tension.[79] Phosphorylation sites in other regions of the titin protein that do not regulate mechanical function also have been identified; some have speculated that these PTMs may regulate docking interactions and influence titin's role as a molecular scaffold.

Myofilament Protein Cleavage

Cardiac injury and oxidative stress also can lead to the degradation of sarcomeric proteins. Early studies showed that cTnI degradation is a prominent feature of ischemic damage, that degraded forms of cTnI remain associated with the myofilament lattice, and that cTnI cleavage may contribute to ischemia-induced changes in force generation and myofibrillar calcium sensitivity.[80,81] Some studies attribute myofilament protein degradation to μ-calpain, a calcium-dependent myofibril-associated protease that is activated in ischemic cardiomyocytes.[82] There is evidence that cTnI is degraded to progressively smaller cleavage products with increasingly severe or prolonged intervals of ischemia/reperfusion injury. A brief episode of ischemia/reperfusion injury leads to the conversion of cTnI (a 210 amino acid protein) to a smaller degradation product (residues 1–193) that forms covalent complexes with cleaved forms of cTnT and cTnC.[83] More severe ischemia/reperfusion injury leads to further degradation of cTnI and the accumulation of shorter catalytic fragments (consisting of residues 63–193 and 73–193) that lack the N-terminal PKA phosphorylation sites and do not form these covalent complexes. Some studies suggest that cTnI may be protected from this form of proteolytic degradation by PKA-dependent phosphorylation of cTnI at Ser^{23}/Ser^{24}.[82,83] cTnT also seems to be vulnerable to calpain-dependent proteolytic cleavage with even very brief episodes of ischemia/reperfusion injury. Calpain cleaves cTnT at a site that removes the NH_2-terminal modulatory domain, leaving a conformationally altered cTnT core structure (residues 72–291) that displays altered binding to cTnI, cTnC, and Tm.[84] Finally, MLC-1 also is degraded during prolonged/severe episodes of ischemia/reperfusion injury; this contributes to a decrease in force generation and calcium sensitivity.[81]

Although most studies have focused on calpain-mediated proteolytic events that are localized to the sarcomere, calpain could in theory influence contractile function by proteolytically activating PKs that phosphorylate myofibrillar proteins.[85] For example, calpain cleaves PKCα at the V3 hinge region, freeing the C-terminal catalytic domain from the autoinhibitory constraints imposed by the N-terminal regulatory domain. There is recent evidence that the PKCα catalytic domain fragment displays a high level of constitutive activity; it acts as a rogue kinase to phosphorylate cellular substrates, including those that are not (or are only weakly) phosphorylated by

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full-length PKCα. Receptor-independent proteolytic activation mechanisms are not specific for PKCα, as calpain cleaves other PKC isoforms[86,87] and other Ser/Thr kinases such as PKD.[88] A role for unregulated/mislocalized catalytic domain fragments generated during oxidative stress, as mediators of pathological cardiac remodeling and changes in contractile performance, has not been considered.

Calpain may not be the only (or even the primary) mediator of sarcomeric protein breakdown in the ischemic heart, as proapoptotic stimuli and oxidative stress also increase the activity of other proteolytic enzymes. For example, caspase-3 is activated by proapoptotic stimuli and it cleaves actin, α-actinin, and cTnT. Caspase-3 cleaves cTnT at a consensus site at DFDD[97], but only when the protein is incorporated into the myofilament lattice; caspase-3 does not cleave free cTnT.[89] Functional studies link caspase-3 treatment of skinned fiber bundles to defects in force/Ca^{2+} relations and myofibrillar ATPase activity. These results suggest that caspase-induced myofilament protein breakdown may contribute to mechanical dysfunction and the evolution of heart failure.[89] However, the importance of caspase-3 as a general mediator of myofibrillar protein breakdown in setting oxidative stress remains uncertain, ascaspase-3 contains a redox-sensitive catalytic cysteine (Cys[163]); oxidative modifications (S-glutathionylation or S-nitrosylation) at this site have been linked to a decrease in caspase-3 activity.[90–92]

Matrix metalloproteinase (MMP)-2 (an abundant MMP in cardiomyocytes and many other cell types) also has recently emerged as a functionally important redox-activated endopeptidase that cleaves sarcomeric proteins. In fact, some have argued that at least certain proteolytic events previously attributed to calpain may actually be mediated by MMP-2, as (1) calpain and MMP-2 cleave many common substrates, (2) many inhibitors (MDL-28170, ALLN, ALLM, and PD-150606) that have been used to define the cardiac actions of calpain are also effective inhibitors of MMP-2,[93] and (3) degraded forms of sarcomeric proteins such as cTnI are not detected in transgenic mice with cardiac-specific calpain overexpression.[94] MMPs are zinc-dependent endoproteinases that are synthesized as latent, inactive zymogens that are maintained in an inactive state by an interaction between a cysteine thiol in the propeptide domain and the Zn^{2+}-containing catalytic domain. MMP-2 is activated in the pericellular or extracellular compartment by upstream proteases (such as MMP-14) that cleave the inhibitory propeptide domain and expose the active site. This leads to the degradation of extracellular matrix and underlies MMP-2 widely recognized roles in tissue remodeling (including embryogenesis, angiogenesis, myocardial infarction, and various forms of wound healing). However, there is recent evidence that the highly conserved Cys in the propeptide domain is a target for oxidative modifications (specifically, $ONOO^-$-dependent S-glutathiolation); an oxidative modification at this site disrupts the intramolecular autoinhibitory interaction and provides a nonproteolytic mechanism to activate MMP-2.[95] The redox-activated form of MMP-2 is recovered in the sarcomere, where it anchors to proteolytic targets such as cTnI and MLC-1. MMP-2 cleaves cTnI, MLC-1, and MLC-2 during ischemia/reperfusion injury; some studies suggest that these sarcomeric protein cleavage events contribute to oxidative stress-dependent defects in cardiac contractility.[96–98] Moreover, there is increasing evidence that the controls of redox-induced events in the sarcomere can be rather elaborate and multi-factorial, as MLC-1 and MLC-2 are primed for MMP-2-dependent degradation by redox-induced PTMs. For example, MMP-2-dependent cleavage of MLC-1 (at $Y^{189}E^{190}$ in its accessible C-terminus) is enhanced by a $ONOO^-$-induced increase in MLC-1-Tyr^{78}/Tyr^{190} nitration and Cys^{81} nitrosylation.[97,99] Similarly, MMP-2-dependent cleavage of MLC-2 is facilitated by nitration at Tyr^{150}.[98] Finally, there is evidence that MMP-2 localizes to the Z-disk where it might play a role in Z-disk assembly and maintenance of sarcomeric integrity by binding and cleaving α-actinin and titin.[100,101]

Conclusions, Caveats, and Future Directions

This article summarizes recent advances in our knowledge of ROS-regulated PTMs in sarcomeric proteins. The lengthy list and spectrum of the redox-regulated events summarized in Table 1 is a testament to recent advances in methodologies for proteomic profiling and the growing recognition that redox biology plays a fundamentally important role in the control of cardiac contraction. Although there is considerable evidence that many protein redox modifications lead to functionally important changes in sarcomeric protein structure, stability, interactivity, and activity, our current understanding of the redox-dependent mechanisms that control contractility in vivo in the intact heart remains rather rudimentary in large part because biochemical studies have focused primarily on redox-dependent modifications on single purified contractile proteins or preparations that contain selected components of the contractile apparatus; the large size and limited solubility of many myofibrillar proteins makes some types of biochemical analysis quite challenging. Extrapolations from these more reductionist systems to the in vivo context may be misleading for several reasons. First, the conformation and exposed surfaces of a contractile protein may be altered by interactions with binding partners in the myofilament lattice in a manner that influences the accessibility of PTM sites and either facilitates or prevents reactivity. Second, ROS-dependent modifications of sarcomeric proteins seldom occur in isolation, and structural modifications of 1 protein can have far-reaching effects on molecular interactions between sarcomeric proteins elsewhere in the complex. Hence, the ensemble effects of all PTMs in the sarcomere determine the nature of the ROS-induced change in cardiac contractility in vivo in the intact heart. Finally, generalizations regarding ROS-dependent changes in cardiac contraction ignore the fact that oxidative stress represents a spectrum of responses that depend on the precise chemical nature of the oxidant species and level/severity of oxidative stress; this review provides numerous examples of oxidant species and ROS-activated enzymes that trigger different (in some cases diametrically opposite) effects on cardiac contractility. The complexities inherent in these redox-regulated mechanisms that control pump function present both

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challenges and opportunities for the development of more specific therapeutic strategies for heart disease.

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Disclosures

None.

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