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Posttranslational Modifications of Myosin Light Chains Determine the Protein Fate

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1. Introduction

The advances in proteomics over the last decade have made it possible for a more detailed study of protein posttranslational modifications. Posttranslational modification of proteins is an important signaling mechanism regulating vital pathways ranging from transcription to translation, in metabolism, cell survival, and cell death. Posttranslational modification of proteins has commonly been associated with the loss/gain of function and signal transduction with the concept of phosphorylation being the hallmark. However, many other posttranslational modifications of proteins have been detected and their implication to overall cellular homeostasis remains to be elucidated.

The cardiovascular system, in particular the heart due to its high metabolic rates, sensitivity to oxidative stress and necessity to adapt quickly to new environments, is an ideal candidate to the study of posttranslational modifications in physiology and pathology. Cardiac contractile function relies significantly on the integrity of its contractile apparatus, with the myosin light chains being important contractile elements. We have recently described the role of nitration and nitrosylation of ventricular myosin light chains (MLCs) on its degradation by the proteolytic enzyme matrix metalloproteinase-2 (MMP-2) (Doroszko et al. 2010; Doroszko et al. 2009; Polewicz et al. 2010). Using distinct experimental models of oxidative stress, such as hypoxia-reoxygenation or ischemia/reperfusion, we have detected pathological nitration and nitrosylation of MLC induced by oxidative stress. According to our findings, nitration and nitrosylation of MLCs is associated with an increased affinity for MMP-2 and a consequent increase in degradation of these proteins that is associated with a worsening in cardiac contractile function during either reoxygenation or reperfusion.

Since contractile dysfunction is a predictor of patient outcome (Antman et al. 2004), it is crucial to understand the mechanisms behind the development of contractile dysfunction. Moreover, the identification of mechanisms that lead to contractile dysfunction can help and result in the development of new therapeutic approaches aiming at preventing and/or treating contractile dysfunction following oxidative stress.

This review will focus on the current knowledge of posttranslational modification of myosin light chain, a cardiac contractile protein, and how these modifications contribute to protection or pathogenesis in the setting of cardiac injury and contractile dysfunction triggered by oxidative stress. Moreover, this review will deal with the importance of posttranslational modifications of proteins and its determination of protein fate.
2. Proteomics

The term "PROTEOME" (PROTEin complement to the genOME), introduced in 1994, has attracted great attention, as approximately 30,000 human genes correspond to several million different gene products (proteins, peptides). The genome is intrinsically static and basically the same in every cell type, while the proteome is highly dynamic, differs between cell types, and does all the work. Proteins are the most common diagnostic and therapeutic targets in medicine, and the search for the proteome may lead to the discovery of new diagnostic and therapeutic targets. Classical proteomics, or what is now referred as "expression profiling", is a process in which total cellular or tissue proteins are separated on 2D gels and the visible protein spots are identified by peptide mass fingerprinting (Dunn 2000; Pandey and Mann 2000). This approach has been used to generate extensive proteomics online databases containing protein data obtained from the hearts of animals with cardiovascular disease states (Arrell et al. 2001a; Arrell et al. 2001b; Evans et al. 1997; Scheler et al. 1999).

The field of proteomics has its roots in the marriage between 2D electrophoresis and mass spectrometry. In most cases, 2-dimensional electrophoresis is used to separate individual proteins and their modified forms, which are then identified and further characterized/analyzed by mass spectrometry. To date, proteomics has identified changes in more than 40 proteins in heart diseases such as dilated cardiomyopathy, varying degrees of I/R injury, and heart failure (Arrell et al. 2001a; Corbett et al. 1998; Foster and Van Eyk 1999; Jager et al. 2002; Jiang et al. 2001; Schwertz et al. 2002).

Proteomics is an ideal approach to elucidate PTMs associated with kinase activity. Positive and negative modulation of heart contractility by short-term phosphorylation reactions at multiple sites in MLC2, TnI, TnT, α-tropomyosin, and myosin binding protein-C, have been known for almost a decade (Schaub et al. 1998). An example of this modification is the discovery of novel phosphorylation of MLC1 in preconditioned cardiomyocytes (Arrell et al. 2001a). However, the role of this PTM is not known. Phosphorylation of MLC1 was also detected in congestive heart failure (CHF) and this was associated with a decreased sensitivity to 8-Br-cGMP-mediated smooth muscle relaxation (Karim et al. 2004). Similarly, three different PTMs were found in functionally important N-terminal sites of MLC2, two occurred in normal hearts (phosphorylation and deamidation) and one (n-terminal truncation) was associated with I/R injury (White et al. 2003). We have found the same PTMs in MLC1 in our model IR with the exception that phosphorylation and deamidation were associated with truncated forms of MLC1. Thus, the use of the proteomics approach to investigate mechanisms underlying heart disease should result in the generation of new therapeutic strategies and the establishment of precise and sensitive diagnostic markers. A schematic representation of a proteomic workflow is given in figure 1.

2.1 Methodology used in the study of myosin light chains posttranslational modifications

Although new advances have been made recently in the development of new technology for protein separation, the proteomic method relies significantly on 2-dimensional electrophoresis (2-DE) for protein separation for further analysis by mass spectrometry. One of the early limitations of the use of 2-DE for sample generation for mass spectrometry analysis was reproducibility. The problem was generated by the fact that gradient gels are difficult to cast consistently and only 2 gels could be run simultaneously. Recent
Posttranslational Modifications of Myosin Light Chains Determine the Protein Fate

Fig. 1. Schematic representation of a proteomic method workflow. Samples are loaded and separated using 2-dimensional electrophoresis (2-DE). Following 2-DE, protein spots of interest are identified and subjected to in-gel tryptic digestion followed by a mass spectrometry protocol (typically LC/MS/MS or MALDI TOF-TOF). Data generated from mass spectrometry can be used to identify the protein using the Mascot search database or, after protein identification, for detection of posttranslational modifications (PTMs) using the ExPASy-FindMod tool (http://web.expasy.org/findmod/findmod_masses.html).

Technological advances gave rise to commercially available pre-cast gels (Criterion pre-cast gels, BioRad, Hercules, CA, USA) and the development of dodeca electrophoresis systems allowing for the simultaneous run of up to 12 gels (Criterion Dodeca Cell, BioRad, Hercules, CA, USA). These advances were very important in the achievement of reproducibility of sample generation by 2-DE.

The majority of the results here described in terms of the study of posttranslational modifications of myosin light chain 1 and 2 were obtained using the following methodology as described by:

Protein samples for 2-DE were prepared by mixing frozen (-80°C), powdered heart tissue (40 to 60 mg wet weight) with 200 µL rehydration buffer (8 mol/L urea, 4% CHAPS, 10 mmol/L DTT, 0.2% Bio-Lytes 3/10 [BioRad, Hercules, CA, USA]) at room temperature. Samples were sonicated for 2X5 seconds and centrifuged (10 minutes at 10,000g) to remove insoluble particles. Protein content of the heart extract in rehydration buffer was measured with the BioRad Bradford protein assay.

Protein samples (400 g) were applied to each of 11 cm immobilized linear pH gradient (5-8) strips (IPG, BioRad, Hercules, CA, USA), with rehydration for 16–18 h at 20°C.
Isoelectrofocusing was performed using the BioRad Protean IEF cell with the following conditions at 20°C with fast voltage ramping: step 1: 15 min with end voltage at 250 V; step 2: 150 min with end voltage at 8000 V; step 3: 35 000 V-hours (approximately 260 min). Following isoelectrofocusing the strips were equilibrated according to the manufacturer’s instructions. The second dimension of 2-DE was performed with Criterion pre-cast gels (8 – 16%) (BioRad). After separation, proteins were detected with Coomassie Brilliant Blue R250 (BioRad). To minimize variations in resolving proteins during the 2-DE run, 12 gels were run simultaneously using a Criterion Dodeca Cell (BioRad, Hercules, CA, USA). Because of this limitation for 2-DE analysis we used 4 hearts from each group. All the gels were stained in the same bath and next scanned with a calibrated densitometer GS-800 (BioRad, Hercules, CA, USA). Quantitative analysis of MLC1 and MLC2 spot intensities from 2-DE were measured with PDQuest 7.1 measurement software (BioRad, Hercules, CA, USA). MLC1 and MLC2 protein spots were manually excised from the 2-DE gel. These spots were then processed using a MassPrep Station (Waters, Milford, MA, USA) using the methods supplied by the manufacturer. The excised gel fragment containing the protein spot was first destained in 200 µl of 50% acetonitrile with 50 mM ammonium bicarbonate at 37°C for 30 minutes. Next, the gel was washed twice with water. The protein extraction was performed overnight at room temperature with 50 µL of a mixture of formic acid, water, and isopropanol (1:3:2, vol:vol). The resulting solution was then analyzed by mass spectrometry (MS). For electrospay, quadruple time-of-flight (Q-TOF) analysis, 1 µl of the solution was used. Liquid chromatography/mass spectrometry (LC/MS) was performed on a CapLC high-performance liquid chromatography unit (Waters, Milford, MA, USA) coupled with Q-TOF-2 mass spectrometer (Waters, Milford, MA, USA). A mass deviation of 0.2 was tolerated and one missed cleavage site was allowed. Resulting values from mass spectrometry (MS/MS) analysis were used to search against the NCBI NR and SwissProt databases with Mammalia specified. We used the Mascot (www.matrixscience.com) search engine to search the protein database. Posttranslational modifications were determined using the ExPASy-FindMod tool (http://web.expasy.org/findmod/findmod_masses.html).

3. Cardiac contractile proteins

The heart is the central organ for the circulatory system and is responsible for providing an efficient flow of blood to the whole body in order to meet the metabolic demands of the organism by delivering oxygen and nutrients and, at the same time, removing metabolic waste. Often seen as a pump, the heart relies on the integrity of its contractile machinery in order to efficiently perform its function. The basic unit of contraction is the sarcomere. The sarcomere is constituted of thick and thin filaments that, during contraction, slide over each other leading to the shortening of the sarcomere and contraction. The thick filament is mainly constituted of myosin while the thin filament is mainly constituted of actin, tropomyosin, and troponins (Figure 2). The interaction between thin and thick filaments, the crucial component for the generation of a contractile force, occurs between actin and the myosin head.

3.1 Myosins

Myosin is a large complex molecule. It consists of two heavy chains, an α-helical tail, and four myosin light chains (Craig and Woodhead 2006; Dominguez et al. 1998; Rayment et al. 1993b). The heavy chains (myosin heavy chain, MHC) have the ATPase activity necessary to
trigger sliding between filaments and the consequent contraction. The two light chains (myosin light chain 1 and 2, MLC1 and MLC2) confer stability to the myosin head and also have actin binding motifs. MLC1 is also referred to as the essential light chain (ELC) and is present in the hinge of the myosin head for stability purposes. MLC2 is also referred as regulatory light chain (RLC) and together with MLC1 forms the hinge region between the globular head and the α-helical tail of myosin.

The essential light chains (ELC) are expressed by three different genes (MYL1, 3 and 4) which give rise to four isoforms of ELC/MLC (Hernandez et al. 2007). The nomenclature adopted depends on the tissue expressed (ELC_a and ELC_v for atrium and ventricular ELC/MLC, respectively) or whether it is full or short MLC (MLC1 and MLC3 for long and short MLCs, respectively) (Hernandez et al. 2007). The nomenclature for myosin light chains is not always obvious and for this manuscript we will refer to MLC1 as the full length myosin light chain present in the sarcomeres of the ventricle.

It has been described that the amino terminus of MLC1 interacts with the carboxy terminus of actin during contraction (Andreev and Bojedo 1999; Efimova et al. 1998; Henry et al. 1985; Milligan et al. 1990; Miyanishi et al. 2002; Morano et al. 1995; Nieznanska et al. 1998; Nieznanska et al. 2002; Niezniakski et al. 2003; Timson et al. 1999; Trayer et al. 1987; VanBuren et al. 1994). This interaction of MLC1 with actin suggests an important role of MLC1 in the regulation of contraction. Indeed, selective removal of MLC1 from the myosin molecule resulted in a reduction of ~50% of the force generated (VanBuren et al. 1994).

The regulatory light chain (RLC), referred as MLC2 in this review, is involved, as the name suggests, in the regulation of contraction. In the heart, two isoforms are found: a ventricular specific (MLC2_v) and an atrium specific (MLC2_a) isoform (Collins 2006). MLC2, together with MLC1, contributes to the mechanical stability of the hinge of the head region of the myosin molecule. MLC2 has been better studied and characterized due to the fact that it can be phosphorylated. MLC2 phosphorylation under basal conditions has been demonstrated to regulate Ca^{2+}-dependent contraction (High and Stull 1980; Mizuno et al. 2008; Stull et al. 1980; Sweeney and Stull 1986).

In order for proper sarcomeric contraction, the myosin structure has to be stable and fine tuned. It is the role of the light chains, present in the hinge of the head region, to assure stability of the head region and fine tune contraction by regulating the interaction between MHC and actin.

4. Posttranslational modifications

Virtually all proteins are subjected to posttranslational modifications. In this text, posttranslational modification will refer to the addition of a chemical group to amino acid residue which has a biological functional. Mass spectrometry can be used to determine peptide masses belonging to the native protein. According to the mass of each peptide one can infer about the presence or absence of a posttranslational modification that has a unique mass signature. A useful tool in determining posttranslational modifications by using peptide masses is ExPaSy-FindMod tool (available at http://web.expasy.org/findmod/findmod_masses.html). Up to date the available information from ExPaSy-FindMod tool, shows seventy one groups of posttranslational modifications that can be detected from analysis of peptide mass fingerprints. Of these, phosphorylation is by far the most studied and well know, mainly due to the identification of enzymes mediating phosphorylation of protein residues: protein kinases.

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Phosphorylation is commonly associated with signal transduction, the hallmark of signaling cascades mediated by kinases. Other posttranslational modifications have recently gained more attention, mainly due to the fact that they are associated with oxidative stress. Protein nitration and nitrosylation are common events occurring in cells subjected to oxidative stress. Contrary to phosphorylation, no enzyme has been described to mediate nitration and nitrosylation and these modifications are often seen as a non-enzymatic posttranslational modification dependent on the presence, identity and concentration of reactive nitrogen species. The role of protein nitration on cellular signal transduction pathways has been reviewed by Yakovlev and Mikkelsen (Yakovlev and Mikkelsen 2010). The authors conclude that the gathered evidence supports the notion of protein nitration being a specific reaction. Though not entirely clear, it appears that nitration of protein residues by reactive nitrogen species is dependent on the tertiary structure of the protein and in particular the chemical environment of the tyrosin residues.

Due to the number of possible posttranslational modifications currently identified and the fact that the same posttranslational modification can occur in different amino acids, it is clear that the study of posttranslational modifications of protein under physiological and pathological conditions is a difficult task. Moreover, posttranslational modifications are not isolated reactions. A protein molecule present in physiological or pathological conditions may have more than one posttranslational modification. Also, the same protein can exhibit different types of posttranslational modifications at one time. Hence, the study of posttranslational modifications of proteins is difficult but also of high importance due to the nature of physiological and pathological consequences these modifications often cause. Also of importance is the fact that the study of posttranslational modification of cardiac contractile proteins can result in the identification of disease-specific markers of heart injury, hence contributing to the development of more sensitive and specific biomarkers of heart injury.

4.1 Biomarkers of heart injury

A biomarker is defined as a reproducibly detectable molecular feature, usually present in an accessible bodily fluid or tissue, that is correlated with a disease state. Cardiac enzymes have long been used as front-line diagnostic tools in the detection of myocardial injury caused by myocardial ischemia. However, the most commonly used enzymes (such as creatine kinase (CK) and its myocardial fraction CK myocardial band (MB), aspartic aminotransferase, and lactate dehydrogenase) are limited in their ability to detect myocardial injury by short diagnosis windows, have limited sensitivities, and lack specificity because of their presence in skeletal muscle. Similarly, myoglobin also lacks specificity because its release from skeletal muscle cannot be distinguished from its release from the heart muscle (Christenson and Azzazy 1998). Thus, there is a need to develop novel biomarkers in order to more effectively treat and diagnose myocardial infarction (MI).

Using the proteomics approach a time-dependent increase of TnI in the serum from patients with MI was reported (Labugger et al. 2000). This new finding led to the suggestion that MLC1, as a contractile protein, could be considered as a new protein biomarker in I/R injury of the heart (Lee and Vasan 2005; Sato et al. 2004). The list of biomarkers in cardiovascular diseases will grow, particularly when the proteomics approach is used. This method has already identified 177 different proteins (including their different molecular forms) with the potential to be good candidates as biomarkers (Anderson 2005) in cardiovascular disease such as stroke.
4.2 MLCs in heart injury
Muscles contract when filaments containing a molecular motor, myosin, pull against another set of filaments containing mainly actin. The source of energy for this directional movement is provided by the hydrolysis of ATP, which is catalyzed by myosin. Muscle myosin is a hexamer consisting of two heavy chains (MHC), two regulatory (or phosphorylatable) light chains (known as MLC2 or RLC) and two essential chains (known as MLC1, and alkali or ELC). The myosin heavy chain is an elongated molecule where more then 90% of the protein is a coiled coil tail formed by the two heavy chains. However, the N-terminus of MHC is globular and contains ATPase activity, the actin binding site, and MLC1 and MLC2 binding sites (Rayment et al. 1993a; Rayment et al. 1993b). The light chains from cardiac and skeletal muscles are not directly involved in the regulation of contraction. However, both MLC1 and MLC2 can exert a subtle modulatory effect.

The precise molecular basis for myocardial stunning remains unknown, but protein damage within the myofilament is a likely mechanism. It is almost certain that stunning is a multifactorial process. One potential target is ventricular MLC2, which via changes in its phosphorylation status, modulates contractile force generation arising from actin-myosin MHC interaction (the structure, function and malfunction of MLC2 have been reviewed by Szczesna, (Szczesna 2003)). Three years ago an Australian group, using an experimental protocol similar to ours, found changes in phosphorylation of MLC2 and showed how these changes are correlated with the function of stunned myocardium (White et al. 2003). In another model, involving pharmacologically preconditioned isolated cardiomyocytes, altered phosphorylation of MLC1 was also found, but the role of this modification is not yet known (Arrell et al. 2001a).

Not only does heart injury induce chemical modification of MLCs, but during acute congestive heart failure entire MLC molecules, or their degradation products, are released into the circulation (Goto et al. 2003; Hansen et al. 2002). Van Eyk and colleagues have shown that the release of degradation products of MLC1 to coronary effluent is positively correlated with the duration of ischemia (Van Eyk et al. 1998). And White and co-workers found that both to MLC1 and MLC2 are released into the effluent of ischemic hearts (White et al. 2003). There was no evidence as to what proteolytic enzyme could be responsible for MLC degradation or what molecular mechanism could account for the release of their products into the circulation. Our work on the degradation of MLC1 in I/R heart shows that MMP-2 is responsible (at least in part) for the degradation of this protein (Doroszko et al. 2009; Polewicz et al. 2010; Sawicki et al. 2005). Although, the mechanism of release is still unknown, it could result from a loss of cell membrane integrity. Despite the many unanswered questions about the molecular basis of I/R injury in the heart, cardiac MLC1 is becoming a very important candidate as a biomarker of heart injury.

4.3 Phosphorylation
Phosphorylation is a posttranslational modification that consists of the addition of a phosphate group to serine (Ser), threonine (Thr) or tyrosine (Tyr). The addition of the phosphate group to these amino acids is catalyzed by kinases. The currently described mechanism of phosphorylation is that it essentially works as a switch, turning the function of phosphorylated protein on or off. Other consequences of protein phosphorylation may involve subcellular localization of proteins, protein-protein interaction, and proteolytic degradation. In fact, our ongoing studies on role of posttranslational modifications in the development of cardiac contractile dysfunction implies that the phosphorylation of MLC1...
During ischemia/reperfusion results in its increase degradation, possibly by MMP-2, contributing to ischemia/reperfusion injury. Phosphorylation of MLC1 has been demonstrated previously (Arrell et al. 2001a) but it has been associated with stability of the myosin head. The authors reported phosphorylation of rat/human Thr 69/64 and Ser 200/194 or 195. Our unpublished data demonstrates that phosphorylation of MLC1 has direct implications in its degradation by MMP-2. We observed \textit{in vitro} phosphorylation (by myosin light chain kinase) of human recombinant MLC1 at Thr127, Thr129 or Tyr 130, as well as Ser179 and Tyr186. In MLC1 from isolated rat hearts subjected to ischemia/reperfusion we observed six phosphorylated residues: Thr69, Thr77 or Tyr78, Thr132, Thr134 or Tyr135, Thr164, Ser184 and Tyr190. Our data suggests a physiological role for MLC1 phosphorylation of Thr69 and Thr132, Thr134 or Tyr135, since these phosphorylations are present in aerobic control hearts, with the remaining four phosphorylations being induced by ischemia/reperfusion (Table 1). The observed phosphorylations of MLC1 induced by ischemia/reperfusion resulted in an increased degradation of MLC1. In an unpublished \textit{in vitro} study we observed that when MLC1 was phosphorylated by the myosin light chain kinase (MLCK), the affinity of MMP-2 for MLC1 was increased and this increase in affinity resulted in an increase in the degradation of MLC1. Taken together, these observations suggest a role for protein phosphorylation in the induction of proteolytic degradation, namely by MMP-2.

| Identified posttranslational modified residues | MLC1 | MLC2 |
|-----------------------------------------------|------|------|
| **Posttranslational Modification**             |      |      |
| **Phosphorylation**                            |      |      |
| \textit{in vitro} (human recombinant)          |      |      |
| Thr127/Thr129/Tyr130, Ser179, Tyr186          |      |      |
| \textit{ex vivo} (rat heart)                    |      |      |
| Thr69, Thr77/Tyr78, Thr132/Thr134/Tyr135, Thr164, Ser184, Tyr190 |      |      |
| **Tyr nitration**                              |      |      |
| \textit{in vitro} (human recombinant)          |      |      |
| Tyr73, Tyr130, Tyr185                           | Tyr152 |      |
| \textit{in vivo} (piglet heart)                 |      |      |
| Tyr141, Tyr118, Tyr152                         |      |      |
| \textit{ex vivo} (rat heart)                    |      |      |
| Tyr78, Tyr190                                  |      |      |
| **Cys S-nitrosylation**                        |      |      |
| \textit{in vitro} (human recombinant)          |      |      |
| Cys67, Cys76                                  |      |      |
| \textit{in vivo} (piglet heart)                 |      |      |
| Cys138                                        |      |      |
| \textit{ex vivo} (rat heart)                    |      |      |
| Cys81                                         |      |      |

Table 1. Identification of MLC1 and MLC2 protein residues subjected to posttranslational modification leading to protein degradation.
To our knowledge these are the first observations concerning phosphorylation of a target protein contributing to the direct proteolytic degradation of that protein. Since it is well known that during several distinct disease processes the activation of phosphorylation cascades occur we speculate that besides up- and down-regulation of protein activity, phosphorylation is responsible for signaling protein degradation contributing directly to the progression of the disease process.

4.4 Nitration and S-nitrosylation

Protein tyrosine nitration has been implicated in many pathological conditions and diseases such as inflammation, chronic hypoxia, myocardial infarction and diabetes among others (Blantz and Munger 2002; Brindici et al. 2010; Donnini et al. 2008; Giasson et al. 2000; Jones et al. 2009; Kang et al. 2010; Koeck et al. 2009; MacMillan-Crow et al. 1996; Naito et al. 2008; Pacher et al. 2007; Pavlides et al. 2010; Pieper et al. 2009; Reyes et al. 2008; Reynolds et al. 2005; 2007; Smith 2009; Upmacis 2008; Zhang et al. 2010). However, a physiological role for protein tyrosine nitration should not be excluded. Not all the tyrosine residues in a protein are targets for nitration either in vitro or in vivo. Moreover, the observed nitratations of tyrosine very seldom coincide between in vitro and in vivo studies. Of importance is the fact that nitration of tyrosine residues is a selective process that appears to be under tight control, even though the exact mechanisms for the regulation of tyrosine nitration remain unknown.

Nitration of protein tyrosine residues (formation of nitrotyrosine) has been suggested to facilitate proteolysis of the nitrated protein (Yakovlev and Mikkelsen 2010). We have recently shown that the contractile proteins MLC1 and MLC2 (part of the thick filament of the sarcomere) are subjected to tyrosine nitration and cysteine s-nitrosylation in cardiac models of oxidative stress (Doroszko et al. 2010; Doroszko et al. 2009; Polewicz et al. 2010). Using an in vivo model of neonatal asphyxia in piglets we have shown that both MLC1 and MLC2 are significantly decreased following hypoxia-reoxygenation (Doroszko et al. 2010; Doroszko et al. 2009). Mass spectrometry analysis for nitration and nitrosylation revealed that MLC1 is S-nitrosylated at Cys 138 and nitrated at Tyr 141. Interestingly, these residues are located at the positions P3 and P1’ of the cleavage site for MMP-2 and hypoxia-reoxygenation was associated with an increase in MMP-2 activity. Also, MLC2 from hearts subjected to hypoxia-reoxygenation was nitrated at Tyr 118 and Tyr 152, while no nitration was observed for the control group (Table 1). These data suggest a pathological role for MLC2 tyrosine nitration associated with hypoxia-reoxygenation. Using human recombinant mutant MLC2, in which the tyrosine residue is replaced with phenylalanine, (Y152F) the in vitro incubation with peroxynitrite as a nitrating agent resulted in the prevention of MLC2 degradation by MMP-2, with no nitration observed at position 152. These observations indicate that although MLC2 has two nitration sites, it is Tyr 152 that mediates the signaling of degradation by MMP-2. MLC1 was also studied in a model of isolated adult rat cardiomyocytes subjected to simulated ischemia. Mass spectrometry analysis revealed nitration of Tyr 190, consistent with what was observed in piglet hearts. However, the Cys in the P3 position of the MMP-2 cleavage site was not S-nitrosylated as observed in MLC1 from piglet hearts. Moreover, MLC1 from rat cardiomyocytes was also nitrated at Tyr 78 and S-nitrosylated at Cys 81. In vitro human recombinant MLC1 was nitrated by peroxynitrite (used as a nitrating agent) at Tyr 73 (corresponding to rat MLC1 Tyr 78) Tyr 185 (corresponding to rat MLC1 Tyr 190), Tyr 140 and S-nytrosilated at Cys 76 (corresponding to rat Cys 81) and Cys 67. In vitro nitrated and S-nitrosylated MLC1 was more susceptible to degradation by MMP-2.
These data support the concept of highly regulated nitration and S-nitrosylation of proteins previously suggested, even though the exact mechanism remains unknown. Moreover, not only these processes are highly specific, they are also tightly associated with pathophysiological consequences. In this case, nitration and S-nitrosylation of protein residues is associated with an increase in its degradation by the proteolytic enzyme MMP-2 both in vitro and in vivo.

Fig. 2. Cartoon representation of our proposed model for regulation of contractile protein fate by posttranslational modifications. Reactive oxygen species (ROS) generated during ischemia/reperfusion or hypoxia-reoxygenation can lead to the direct nitration/S-nitrosylation of tyrosine and cysteine residues of MLC1 and MLC2. Also, ROS can lead to the phosphorylation of MLC1 and MLC2.
5. Conclusion

With the development of proteomics technology over the last two decades, more and more information about protein posttranslational modifications has been gathered. The difficulty of studying posttranslational modification of proteins and their physiological and pathological consequences lies on the fact that often (if not always) a protein will exhibit more than one type of posttranslational modification at any given time or more than one posttranslational modification of the same type. Classically, enzymatic production of a certain product, from a given substrate, was limited by the enzyme activity. Also, posttranslational modification of the enzyme, such as phosphorylation, is a valid process to increase enzyme activity. We propose a new paradigm in the regulation of enzymatic activity by modification of proteins previously resistant to degradation. Here we have described the role of nitrosylation, nitration and phosphorylation of cardiac contractile proteins, as substrates for enzymatic reaction, in models of oxidative stress which result in their increased degradation by a proteolytic enzyme (MMP-2) both in vitro and in vivo (Figure 2).

It has been described that posttranslational modification of MMP-2 triggered by oxidative stress can activate the enzyme (Viappiani et al. 2009). Although this may be the case in the in vivo and ex vivo models, the same observations were made in in vitro experiments in which MMP-2 is not posttranslational modified. This new paradigm, that posttranslational modification determine fate of proteins, is an important advance in the understanding of the molecular mechanisms by which oxidative stress can trigger cardiac contractile dysfunction in pathological processes such as ischemia/reperfusion and hypoxia-reoxygenation. activation of MLCK and phosphorylation of MLC1. These posttranslational modifications increase the affinity of MMP-2 for MLC1 and MLC2. MMP-2 degrades MLC1 and MLC2 leading to cardiac contractile dysfunction.

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