Ischemia induced peroxynitrite dependent modifications of cardiomyocyte MLC1 increases its degradation by MMP-2 leading to contractile dysfunction

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Abstract

Damage to cardiac contractile proteins during ischemia followed by reperfusion is mediated by reactive oxygen species such as peroxynitrite (ONOO−), resulting in impairment of cardiac systolic function. However, the pathophysiology of systolic dysfunction during ischemia only, before reperfusion, remains unclear. We suggest that increased ONOO− generation during ischemia leads to nitration/nitrosylation of myosin light chain 1 (MLC1) and its increased degradation by matrix metalloproteinase-2 (MMP-2), which leads to impairment of cardiomyocyte contractility. We also postulate that inhibition of ONOO− action by use of a ONOO− scavenger results in improved recovery from ischemic injury. Isolated rat cardiomyocytes were subjected to 15 and 60 min. of simulated ischemia. Intact MLC1 levels, measured by 2D gel electrophoresis and immunoblot, were shown to decrease with increasing duration of ischemia, which correlated with increasing levels of nitrotyrosine and nitrite/nitrate. In vitro degradation of human recombinant MLC1 by MMP-2 increased after ONOO− exposure of MLC1 in a concentration-dependent manner. Mass spectrometry analysis of ischemic rat cardiomyocyte MLC1 showed nitration of tyrosines 78 and 190, as well as of corresponding tyrosines 73 and 185 within recombinant human cardiac MLC1 treated with ONOO−. Recombinant human cardiac MLC1 was additionally nitrosylated at cysteine 67 and 76 corresponding to cysteine 81 of rat MLC1. Here we show that increased ONOO− production during ischemia induces MLC1 nitration/nitrosylation leading to its increased degradation by MMP-2. Inhibition of MLC1 nitration/nitrosylation during ischemia by the ONOO− scavenger FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), or inhibition of MMP-2 activity with phenanthroline, provides an effective protection of cardiomyocyte contractility.

Keywords: ischemia • MLC1 • peroxynitrite • nitrotyrosine • nitration • nitrosylation • MMP-2 • cardiomyocytes

Introduction

Ischemia/reperfusion (I/R) injury consists of a complex series of processes ranging from metabolic to morphological and contractile adaptations in response to ischemia and/or reperfusion. However, because most observations are made after reperfusion, the weight of each phenomenon to the development of lethal injury remains a matter of controversy (reviewed in [1]).

The role of peroxynitrite (ONOO−) in cardiac I/R injury has been extensively studied and there is evidence showing a burst in ONOO− generation at the beginning of reperfusion [2]. Even though an increase in generation of nitric oxide and superoxide anion, substrates for ONOO− synthesis, has been shown during ischemia [3–4], there is no evidence for increased ONOO− generation before beginning of the reperfusion phase. ONOO− is a highly reactive oxidant which is generated from the coupling
between nitric oxide and superoxide. Its detrimental action on the development of cardiac I/R injury, as well as its negative effects on cardiac systolic function, has been well established [5–10]. Nonetheless, the exact mechanisms by which ONOO− modulates I/R injury and systolic dysfunction remain to be explained.

The nitration of cardiac proteins, including myofilament components such as troponin T and I, α-actin, and myosin light chain 1 (MLC1), during I/R has been previously described [11–16]; however, the mechanism and molecular consequences of this modification remain unknown. Recent studies by Kanski and colleagues have shown that MLC1, a protein of the muscle contractile machinery, can be nitrated in cardiac [13] and skeletal muscle [17] due to biological aging, but the consequence of this modification has not been established. In addition, it has been well documented that myocardial MLC1 is degraded by matrix metalloproteinase-2 (MMP-2) during I/R [18] and hypoxia/reoxygenation (H/R) [19]. MMP-2 is expressed ubiquitously in the heart, including the cardiac myocyte, and has been implicated in a variety of cardiovascular diseases [20–22]. MMP-2 exhibits both extracellular activity (degrading and remodelling of extracellular matrix) and intracellular activity, namely the degradation of myofibrillar proteins [23–25]. Our most recent study on H/R, using an animal model of neonatal asphyxia, showed that the degradation of MLC1 by MMP-2 is associated with post-translational modifications (PTMs) of MLC1 such as tyrosine nitration and cysteine S-nitrosylation [19]. This study suggested that nitration and/or nitrosylation might play an important regulatory role in controlling intracellular proteolytic action of MMP-2 in MLC1 degradation.

Based on our previous work we suggest that ischemia induces ONOO−-dependent MLC1 nitration/nitrosylation and that these modifications, together with increased intracellular MMP-2 activity, lead to increased degradation of MLC1 within the cardiomyocyte.

Materials and methods

This investigation conforms to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

Preparation of cardiomyocytes and simulated ischemia protocol

Myocyte isolation

Adult male Sprague–Dawley rats were anaesthetized with sodium pentobarbital (150 mg/kg i.p.) and hearts were removed. Right ventricular myocytes (VMs) were obtained by enzymatic dissociation as previously described [26–27]. After isolation the cells were suspended in perfusion buffer [2.6 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 60 mM NaCl, 2.5 mM KCl, 1.1 mM MgCl₂, 2 mM CaCl₂, 2.8 mM glucose, 5 mM Taurine], enriched with 5% CO₂, 95% O₂.

VMs were used as we have found that they are a more suitable cell type to use for the experiments we performed for two reasons. First, it is much easier to obtain a consistently high yield of viable live right VMs compared to left VMs. The enzymatic dissociation of VMs from the thicker left ventricle results in a much higher proportion of dead cells, over-digested cells or ‘groups’ of under-digested cells. Second, right VMs are more homogenous in their properties compared to left VMs that are isolated from the endo, mid and epi layers of the left ventricle [28].

Simulated ischemia

Ischemia was induced by covering the cell pellets with a thin layer of mineral oil. This method is well established as a biological model of ischemia using isolated cardiomyocytes and is reviewed in [29]. At 15, 60 and 120 min. of incubation (n = 4 in each group) mineral oil was removed and the cells were rapidly frozen in liquid nitrogen. The viability of cardiomyocytes after ischemia was assessed by trypan blue exclusion test [29–31]. The control group was kept exposed to atmospheric air for 60 min. at 37°C and then frozen.

Experiments in which FeTPPS, a scavenger of ONOO− [32] (5,10,15,20-tetrakis-(4-sulfonatophenyl)-porphyrinato-iron(III)) (Frontier Scientific, Logan, UT, USA), was present were done as follows: after ischemia and stabilization, cardiomyocytes were incubated with FeTPPS (100 μM) for 10 min. As a negative control for FeTPPS, 100 μM of TPPS was used. After incubation, the cells were divided into equal suspension volumes and either subjected to ischemia as described above or maintained in aerobic conditions for control groups.

Similar to the experiments performed with FeTPPS, phenanthroline was added to myocyte suspensions 10 min. before the onset of ischemia at a final concentration of 100 μM. Because phenanthroline has poor solubility in water, a stock solution of 0.2 M was prepared in 100% ethanol and then diluted so that the final concentration of ethanol in the myocyte suspension was 5 × 10⁻⁴ (v/v) and of phenanthroline 100 μM.

For assessment of the effect of ischemia on cardiomyocyte contractility, a 100 μl fraction of the cardiomyocyte suspension was placed on a glass cover slip mounted on an inverted microscope (Nikon, Tokyo, Japan). After a stabilization period the chamber was perfused with oxygenated buffer at a constant temperature of 37°C. Pacing was induced at 1 Hz and an amplitude of 5 V (IonOptix MyoPacer, Milton, MA, USA). Contractile function was measured using a side-mounted IonOptix MyoCam and the IonWizard 6.0 software. An average of three to five cells per fraction was analysed for 10 min. for determination of contractile function.

Preparation of myocyte extracts

Protein samples for 2D electrophoresis (2-DE) were prepared by mixing frozen cardiomyocytes (30 mg wet weight) with 120 μl of rehydration buffer [8 M urea, 4% 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM dithiothreitol (DTT), 0.2% Bio-Lytes 3/10 (BioRad, Hercules, CA, USA)] at room temperature. Samples were sonicated twice for 5 sec. and centrifuged for 10 min. at 10,000 × g at room temperature to remove any insoluble particles. Protein content of the heart extract in rehydration buffer was measured using the Bradford protein assay (BioRad).

For other biochemical studies frozen cells were homogenized on ice in 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin and 0.1% Triton X-100. Homogenates were then centrifuged at 10,000 × g at 4°C for 10 min. and the supernatant was collected and stored at −80°C until further use.
Preparation of recombinant human cardiac MLC1

The cDNA clone for the human ventricular MLC1 (NCBI # NM_000258) was isolated with a two-tube RT-PCR method and Omniscript RT kit (Qiagen, Germantown, MD, USA) using total adult human heart RNA (Stratagene, La Jolla, CA, USA), Oligo dTs (Promega, Madison, WI, USA) and MLC1 specific primers. The sequence of MLC1 wild type (MLC1-WT) clone was verified and confirmed. MLC1-WT DNA was used to transform BL21 (DE3) Codon Plus competent cells (Stratagene). The MLC1 protein was expressed in 8 l of enriched media consisting of 30 g of peptone/l, 20 g of yeast extract/l and 10 g/l of M9 minimal salts with 20 µg/ml of ampicillin and purified using column chromatography (S-Sepharose, DEAE-Sepharcel). The fractions of protein purity 97–99% were pooled and stored frozen at −80°C until needed.

Two-dimensional polyacrylamide gel electrophoresis

Protein (100 µg) was applied to each of 11 cm immobilized linear pH gradient (5–8) IEF strips (BioRad), with rehydration for 16–18 hrs at 20°C. For isoelectrofocusing (IEF), the BioRad Protein IEF cell was used 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/hrs (approximately 260 min.). After IEF, the strips were equilibrated according to the manufacturer’s instructions. A second dimension of 2-DE was then carried out with Criterion pre-cast gels (8–16%) (BioRad). After separation, proteins were stained with Silver Stain plus kit (BioRad). To minimize variation in staining all gels were stained in the same bath. The reproducibility of 2-DE and quality of protein loading has been previously verified by us [18, 33, 34]. Developed gels were scanned using a GS-800 calibrated densitometer (BioRad). Quantitative analysis of MLC1 protein spots’ intensities from 2-DE gels were measured using PDQuest 7.1 software (BioRad).

Mass spectrometry

MLC1 protein spots were manually excised from the 2-DE gel. The spots containing rat cardiac MLC1 protein (2-DE) and preparations of human cardiac MLC1 protein from the in vitro study were processed using a MassPrep Station from Micromass using the methods supplied by the manufacturer. Mass analysis of the trypsin digest of MLC1 was performed on MALDI-TOF Voyager DE-Pro from Applied Biosystems (Carlsbad, CA, USA) and ESI Q-TOF Ultima Global (Waters, Milford, MA, USA) using total adult human heart RNA (Qiagen, Germantown, MD, USA) using total adult human heart RNA (Qiagen, Germantown, MD, USA) as a template. The cDNA clone for the human ventricular MLC1 (NCBI # NM_000258) was isolated with a two-tube RT-PCR method and Omniscript RT kit (Qiagen, Germantown, MD, USA) using total adult human heart RNA (Stratagene, La Jolla, CA, USA), Oligo dTs (Promega, Madison, WI, USA) and MLC1 specific primers. The sequence of MLC1 wild type (MLC1-WT) clone was verified and confirmed. MLC1-WT DNA was used to transform BL21 (DE3) Codon Plus competent cells (Stratagene). The MLC1 protein was expressed in 8 l of enriched media consisting of 30 g of peptone/l, 20 g of yeast extract/l and 10 g/l of M9 minimal salts with 20 µg/ml of ampicillin and purified using column chromatography (S-Sepharose, DEAE-Sepharcel). The fractions of protein purity 97–99% were pooled and stored frozen at −80°C until needed.

In vitro nitration/nitrosylation and degradation of MLC1 by MMP-2

Because rat cardiac MLC1 (accession number: P16409) is commercially unavailable, we used recombinant human cardiac MLC1 (accession number: P08590) for in vitro degradation of MLC1 by MMP-2. Using the LALIGN peptide comparison program (www.ch.embnet.org/software/ LALIGN_form.html), the primary sequence of rat cardiac MLC1 was compared to human cardiac MLC1 showing 93.5% identity.

Recombinant human cardiac MLC1 was used for assessment of the ONOO− effect on in vitro degradation of MLC1 by MMP-2. The MLC1 (12 µg) was pre-incubated with ONOO− (0.01 mM, 0.1 mM and 1 mM) for 30 min. at room temperature. This was followed by incubation with 200 ng of MMP-2 (Calbiochem, Darmstadt, Germany) in 50 mM Tris-HCl buffer containing 5 mM CaCl2 and 150 mM NaCl (total volume 40 µl) at 37°C for 60 min., which corresponds to 60 min. of ischemia. The reaction mixtures were analysed by 12% SDS-PAGE under reducing conditions and visualized by the Coomassie Brilliant Blue G-250 staining method. Developed gels were scanned using a GS-800 calibrated densitometer (BioRad). The degradation of MLC1 was calculated using Quantity One 4.6 software (BioRad).

Examination of experimental peptide mass fingerprinting for nitration and nitrosylation

As an additional method for detection of protein modification, the FindMod tool (2007) was used to find ONOO− related PTMs in the experimental tryptic peptides of MLC1 (http://au.expasy.org/tools/findmod/). Two types of PTMs were considered: nitration of tyrosine (Y) and S-nitrosylation of cysteine (C) with the assumption that up to three PTMs can exist in one tryptic peptide.

Measurement of MMP-2 activity

Gelatine zymography was performed as described [36]. Briefly, homogenates from myocyte preparations containing 30 µg of protein were applied to 8% polyacrylamide gel copolymerized with 2% gelatine. After electrophoresis, gels were rinsed three times for 20 min. in 2.5% acetic acid buffer and 50 µl of pronase (1 mg/ml in acetate buffer) followed by 50 µl of the supernatant to 25 µl of sodium acetate buffer and 50 µl of pronase (1 mg/ml in acetate buffer) followed by heating at 50°C for 18 hrs. After digestion, samples were dried in a Speed Vac system and the extract was dissolved in acetonitrile. Derivatization with 4-fluoro-7-nitrobenzene-2-oxa-1,3-diazole was performed. A 1 µl aliquot was used for HPLC quantification as described [37].

Measurement of nitrotyrosine levels

Nitrotyrosine level, although not a specific marker of ONOO−, has been the hallmark for the evaluation of the effects of ONOO− on protein modification. Nitrotyrosine and tyrosine concentrations in cardiomyocyte homogenates were determined by high pressure liquid chromatography (HPLC) as previously described [37–39]. Briefly, isolated adult cardiomyocytes were sonicated in 400 µl of sodium acetate (10 mM, pH 6.5) and vortexed (1 hr). Samples were then centrifuged at 12,000 × g for 10 min. and a 50 µl aliquot was removed and used for a protein assay according to the Bradford method. Proteolysis was performed by adding 150 µl of the supernatant to 25 µl of sodium acetate buffer and 50 µl of pronase (1 mg/ml in acetate buffer) followed by heating at 50°C for 18 hrs. After digestion, samples were dried in a Speed Vac system and the extract was dissolved in acetonitrile. Derivatization with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole was performed. A 10 µl aliquot was used for HPLC quantification as described [37].

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Immunoblot analysis

Rat cardiac MLC1 and nitrotyrosine level was determined by immunoblot analysis. Protein (30 µg) from isolated cardiomyocyte homogenate was separated using 12% SDS-PAGE [40] and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). MLC1 was identified using polyclonal anti-MLC1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Nitrotyrosine was identified using rabbit polyclonal anti-nitrotyrosine antibody (Cell Signaling Technology, Beverly, MA, USA). Band densities were measured using GS-800 calibrated densitometer and Quantity One 4.6 software.

Immunoprecipitation

Immunoprecipitation of MMP-2 and rat cardiac MLC1 was performed as previously described [19]. Briefly, 300 µg of protein from isolated cardiomyocyte homogenates were incubated with 12 µg of rabbit anti-MMP-2 IgG overnight at 4°C. Unrelated IgG was used as an internal negative control. The immunoprecipitates were analysed by immunoblot for MLC1 level.

Statistical analysis

ANOVA was used to compare the differences between the groups. Post hoc analysis was performed with the Tukey–Kramer multiple comparisons test. Analysis of spot densities of the 2-DE gels was carried out by the Mann–Whitney U-test and Student’s t-test which are incorporated in the PDQuest measurement software. A P-value of less than 0.05 indicated statistically significant differences. Data are expressed as mean ± S.E.M.

Results

Effect of duration of ischemia on cardiomyocyte viability and contractility

After isolation, cardiomyocytes were subjected to 15, 60 or 120 min. of ischemia. In order to determine the times of ischemia for this study we evaluated cell viability at the end of the different ischemic times. After 15 min. of ischemia, cell viability and contractility were similar to control (Fig. 1). Sixty minutes of ischemia significantly reduced cell viability but to a lesser extent than 120 min. of ischemia (Fig. 1A). To further validate our model, we measured contractility of isolated rat cardiomyocytes. The goal of this experiment was to determine the contractile status of isolated cardiomyocytes after different times of ischemia. As seen in panel B (Fig. 1), peak shortening did not change after 15 min. of ischemia but was significantly reduced after 60 min. of ischemia. After 120 min. of ischemia peak shortening was further reduced. Because very low viability and severe impairment of contractility at 120 min. of ischemia were observed, we decided in our study to analyse cardiomyocytes from 15 and 60 min. ischemia groups. Fifteen minutes represents a mild ischemia with no significant cell death and 60 min. represents an acute ischemic episode.

Peroxynitrite formation during ischemia in cardiomyocytes

The production of ONOO⁻ was determined by measuring the levels of nitrotyrosine by HPLC and by immunoblotting in cardiomyocytes subjected to simulated ischemia and compared to the aerobic (control) myocytes. Analysis of the nitrotyrosine to tyrosine ratio showed a 2-fold increase in nitration of tyrosine during 60 min. of ischemia in comparison to the control group (Fig. 2A). Immunoblot analysis (Fig. 2B) showed association of increased levels of nitrotyrosines with increasing duration of ischemia. Increased nitration of tyrosine in the protein band corresponding to MLC1 standard (panel a) in both ischemic groups versus the control group (panel b) was observed.
Analysis of MLC1 level and MMP-2 activity in cardiomyocytes

Two-dimensional polyacrylamide gel electrophoresis analysis of the MLC1 level in cardiomyocyte homogenates is presented in Figure 3A. As shown, the MLC1 level was significantly decreased in cells subjected to 60 min. of ischemia. Similarly, a significant reduction of the MLC1 level was observed by immunoblot analysis (Fig. 3B).

The specific activities of the 72 and 62 kD isoforms of MMP-2 in myocyte homogenates from control and ischemic groups are illustrated in Figure 4A. A significant, progressive increase in total MMP-2 activity was observed after 15 and 60 min. of ischemia compared to control.
Immunoprecipitation of MMP-2 protein with anti-MLC1 IgG followed by immunoblotting for MLC1 demonstrated that MLC1 co-localizes with MMP-2 and that the amount of the MMP-2-MLC1 complex (enzyme substrate) increases with duration of ischemia (Fig. 4B).

**Effect of ONOO⁻ on the degradation of recombinant human cardiac MLC1 by MMP-2 in vitro**

After 60 min. of incubation, degradation of human cardiac recombinant MLC1 was observed in all samples containing MMP-2, independent of pre-incubation of MLC1 with ONOO⁻. Quantitative analysis showed that the degradation of MLC1 was significantly potentiated by pre-incubation with increasing concentrations of ONOO⁻ (Fig. 5A). A high negative correlation ($r = -0.999$) between ONOO⁻ concentration and the MLC1 level was observed (Fig. 5B).

Mass spectrometry analysis for nitrations/nitrosylations within MLC1 peptide obtained from cardiomyocytes

In both, the 15 and 60 min. ischemic groups nitrations of tyrosines 78 (Y78) and 190 (Y190) of rat cardiomyocyte MLC1 were observed. Additionally, nitrosylation of cysteine 81 (C81) was observed in MLC1 but only in myocytes subjected to 60 min. of simulated ischemia (Fig. 6).

Mass spectrometry analysis of ONOO⁻ treated recombinant human cardiac MLC1 from *in vitro* studies revealed that the tyrosine residues 73 (Y73) and 185 (Y185) were nitrated (Fig. 5). In addition, tyrosine 130 (Y130) was nitrated in human MLC1
Human cardiac MLC1 was nitrosylated at cysteine 67 (C67) and cysteine 76 (C76) corresponding to C81 of rat cardiac MLC1. We did not observe the nitrosylation of rat cysteine corresponding to human C67 due to its substitution by glycine 72 (G72) (Fig. 6).

Cardiomyocyte viability and function after simulated ischemia

After 15 min. of ischemia, cell viability was similar to control, and after 60 min. of ischemia cell viability significantly reduced (Fig. 1). To further validate our model we measured contractility of isolated rat cardiomyocytes. The goal of this experiment was to determine the contractility of isolated cardiomyocytes after ischemia. As seen in Figure 7 three parameters were analysed in order to determine mechanical function of isolated myocytes: peak shortening, maximal velocity of shortening and maximal velocity of relengthening. As indicated, the three parameters did not change significantly after 15 min. of ischemia but were significantly reduced after 60 min. of ischemia (Fig. 7).

Effect of treatment with selective ONOO− scavenger (FeTPPS) and MMP-2 inhibitor (phenanthroline) on cardiomyocyte contractile function

Pre-treatment of isolated cardiomyocytes with 100 μM of FeTPPS, a scavenger of ONOO−, prevented impairment of contractile
Fig. 7 Evaluation of contractile function of isolated rat cardiomyocytes subjected to simulated ischemia. Freshly isolated rat cardiomyocytes were either maintained in aerobic conditions or subjected to 15 or 60 min. of simulated ischemia. In parallel, groups were pre-treated with 100 μM FeTPPS, a scavenger of ONOO⁻, for 10 min. prior to the onset of ischemia. Results are presented as percentage of aerobic control values. (A) Function expressed as peak shortening. (B) Function expressed as maximal velocity of myocyte shortening. (C) Function expressed as maximal velocity of myocyte relengthening. Data are expressed as mean ± S.E.M., n = 4/group; *P < 0.05 versus aerobic control, #P < 0.05 versus 60 min. ischemia.

function, as assessed by peak shortening, maximal velocity of shortening and maximal velocity of relengthening when compared to control (Fig. 7). Similarly, pre-treatment of isolated cardiomyocytes with 100 μM of the MMP-2 inhibitor phenanthroline resulted in a significant increase in peak shortening and maximal velocity of shortening after 60 min. of ischemia, in comparison to ischemia alone (Fig. 8). Because the vehicle for phenanthroline was ethanol, aerobic cardiomyocytes were treated with 5 × 10⁻⁴% (v/v) ethanol and function was assessed. Ethanol pre-treatment for 10 min. did not affect function in comparison to non-treated cardiomyocytes, both aerobic or those subjected to 60 min. ischemia.
Fig. 8 Evaluation of contractile function of isolated rat cardiomyocytes subjected to simulated ischemia with phenanthroline treatment. Freshly isolated rat cardiomyocytes were either maintained in aerobic conditions or subjected to 60 min. of simulated ischemia. In parallel, groups were pre-treated with 100 μM phenanthroline, an MMP-2 inhibitor, for 10 min. prior to the onset of ischemia. Results are presented as percentage of aerobic control values. (A) Function expressed as peak shortening. (B) Function expressed as maximal velocity of myocyte shortening. (C) Function expressed as maximal velocity of myocyte relengthening. Data are expressed as mean ± S.E.M., n = 4/group; *P < 0.05 versus aerobic control, #P < 0.05 versus 60 min. of ischemia.
Effect of treatment with selective ONOO\(^-\) scavenger (FeTPPS) and MMP-2 inhibitor (phenanthroline) on MLC1 level

Immunoblot analysis showed that pre-treatment of isolated rat cardiomyocytes with 100 \(\mu\)M of FeTPPS (Fig. 9) or with 100 \(\mu\)M of the MMP-2 inhibitor phenanthroline (Fig. 10) resulted in protection of ischemic-induced decreased MLC1 level.

Discussion

This is the first study to demonstrate the importance of the ONOO\(^-\)-MLC1-MMP-2 axis in heart injury during ischemia. We show that during ischemia there is an increase in the generation of ONOO\(^-\) which induces MLC1 PTMs, such as nitration and nitrosylation, and that these modifications are responsible for increased degradation of MLC1 and impaired myocyte contractility.

The results from our study indicate a novel mechanism of intracellular regulation of MLC1 degradation during ischemia, which is based on ONOO\(^-\)-dependent changes in its susceptibility...
to MMP-2-dependent degradation. Analysis of tyrosine nitration by immunoblotting as well as by HPLC has shown an increase in ONOO\(^-\) production in cells subjected to simulated ischemia compared to control aerobic cells. Mass spectrometry analysis of MLC1 from the in vivo study showed nitration of tyrosines 78 and 190, as well as nitrosylation of cysteine 81 in the rat cardiac MLC1 protein, which were also present in homologous amino acid residues within human cardiac MLC1 used in the in vitro study. Because tyrosine 190 is located in P1' position of the cleavage site for MMP-2 (Fig. 5), the modification of this amino acid residue by ONOO\(^-\) may explain the increased MLC1 degradation by MMP-2.

The role of other modifications remains unclear; however, modifications of tyrosine 78 and cysteine 81 might indicate a new proteolytic site for MMP-2 action within the MLC1 molecule. In contrast to results from studies on hypoxia-reoxygenation [19] we did not detect nitrosylation of cysteine 187 of rat MLC1, which corresponds to cysteine 138 of porcine skeletal MLC1, located in the P3 position in the cleavage site for MMP-2. The lack of nitrosylation of cysteine 187 in rat cardiac MLC1 was most likely related to experimental model differences or conformational changes in the MLC1 molecule that could be species specific.

Our previous reports [18–19, 21, 41] suggest that studying PTMs of MLC1 (including ONOO\(^-\)-related modifications) and its subsequent degradation by MMP-2 may provide new information regarding the mechanisms of ischemia-induced MMP-2-dependent degradation of intracellular proteins. Of importance is the concept of ONOO\(^-\) scavenging by incubation of cardiomyocytes with agents neutralizing ONOO\(^-\) deleterious actions such as FeTPPS. Pre-treatment with FeTPPS protected MLC1 against ischemia-induced degradation by MMP-2. Therefore the use of a specific ONOO\(^-\) scavenger in clinical practice may provide better cardiac protection against ischemic injury. These data are consistent with another study in which FeTPPS conferred protection of cardiac function against cytokine-induced injury [32]. Furthermore, phenanthroline, which is an inhibitor of MMP-2 also protected contractile function and this protection was associated with preservation of the protein levels of intact MLC 1.

These results may lead to the development of new therapeutic strategies for the treatment of ischemic injury. Future pharmacological targets may include the inhibition of nitration and nitrosylation of MLC1 and possibly other protein targets together with inhibition of MMP-2 action. Inhibition of both targets might contribute to better synergistic cardiac protection following myocardial infarction.

In summary we conclude that: (i) ischemia is associated with increased generation of ONOO\(^-\), (ii) ONOO\(^-\) causes nitration and nitrosylation of MLC1 which enhances its degradation by MMP-2 during ischemia and (iii) administration of a ONOO\(^-\) scavenger results in better recovery of viability and contractile function after ischemia.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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