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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 inhition 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

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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 injury [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 $MgCl_2$, 2 mM $CaCl_2$, 2.8 mM glucose, 5 mM Taurine], enriched with 5% CO_2, 95% O_2. Right 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 homogeneous 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 isolation 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 $10,000 \times g$ 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 $10,000 \times g$ 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 dT_{15} (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 select 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-Sephacel). 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) IPG strips (BioRad), with rehydration for 16–18 hrs at 20°C. For isoelectrofocusing (IEF), the BioRad Protean 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). A mass deviation of 0.5 was tolerated and one missed cleavage site was allowed. Resulting values from mass spectrometry analysis for monoisotopic peaks were used to search the NCBInr and Swiss-Prot databases for *Rattus norvegicus* or *Homo sapiens*. We used the Mascot (*www.matrixscience.com*) search engine to identify the protein and to detect protein nitration and nitrosylation. The Mowse scoring algorithm [35] was used to justify accuracy of MLC1 protein identification which is incorporated in the Mascot search engine.

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 $CaCl_2$ 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 mg/ml gelatine. After electrophoresis, gels were rinsed three times for 20 min. in 2.5% Triton X-100 to remove SDS. The gels were then washed twice in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl and 0.05% NaN_3) for 20 min. at room temperature and incubated in incubation buffer at 37°C for 24 hrs. The gels were stained using 0.05% Coomassie Brilliant Blue G-250 in a mixture of methanol : acetic acid : water (2.5 : 1 : 6.5, v : v : v) and destained in aqueous solution of 4% methanol : 8% acetic acid (v : v). Developed gels were scanned with GS-800 calibrated densitometer and MMP-2 activity was measured using Quantity One 4.6 software.

Measurement of nitrotyrosine levels

Nitrotyrosine level, although not a specific marker of $ONOO^-$-dependent tyrosine nitrosylation, 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-nitrobenzeno-2-oxa-1,3-diazole was performed. A 10 μl aliquot was used for HPLC quantification as described [37].

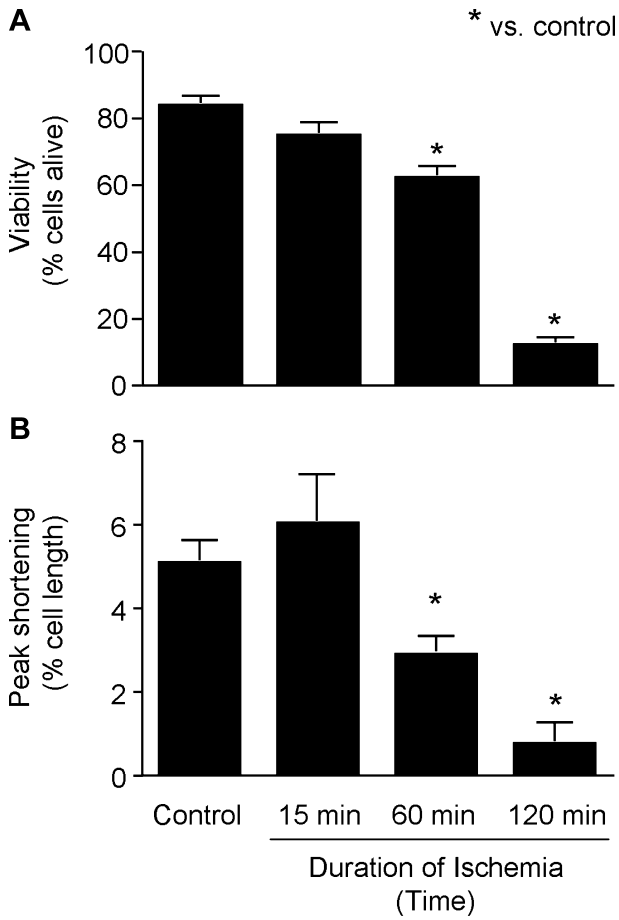


Fig. 1 Effect of duration of ischemia on cardiomyocyte viability and contractility. (**A**) Cell viability after simulated ischemia. Viability was defined as the percentage of live cells. (**B**) Cell contractility (peak shortening) was measure with stimulation at 1 Hz, 5 V. Results are presented as percentage of total cell length. Data are means ± S.E.M., $n = 4$, $^*P < 0.05$.

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.

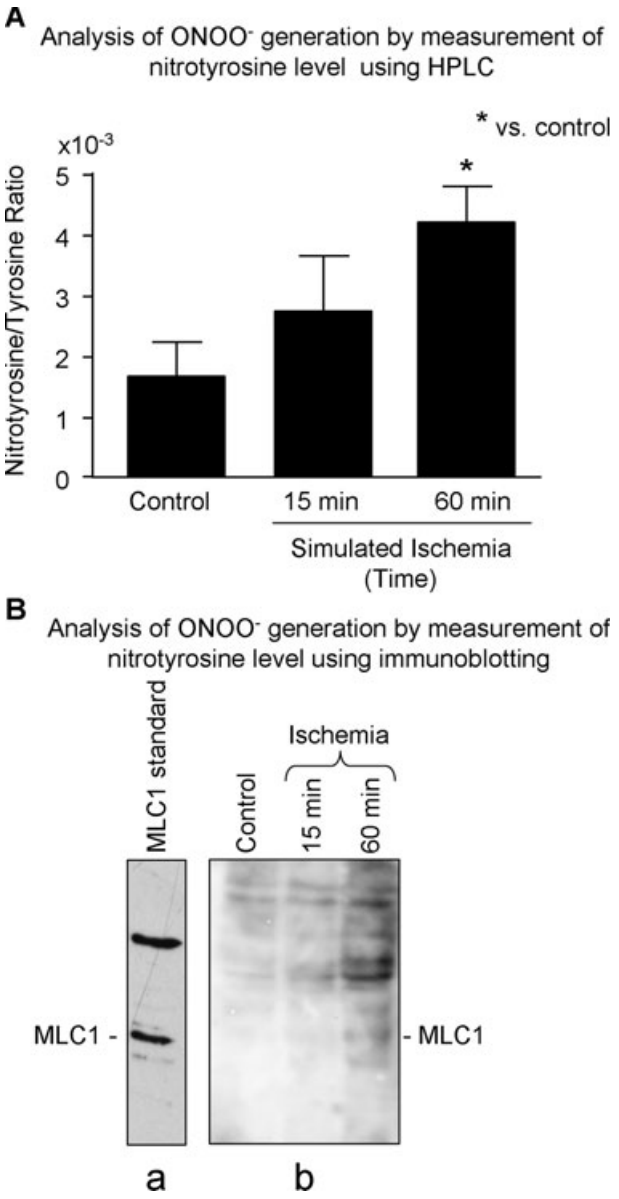


Fig. 2 Nitrotyrosine levels in isolated cardiomyocytes subjected to 15 and 60 min. of ischemia. (**A**) Nitrotyrosine level analysis by HPLC. The $ONOO^-$ production level is shown as a ratio of nitrotyrosine to tyrosine. (**B**) Detection of $ONOO^-$ production by measurement of nitrotyrosine (marker $ONOO^-$ production) using immunoblot analysis with anti-nitrotyrosine antibody. Data are expressed as mean ± S.E.M., $n = 4$/group; $^*P < 0.05$.

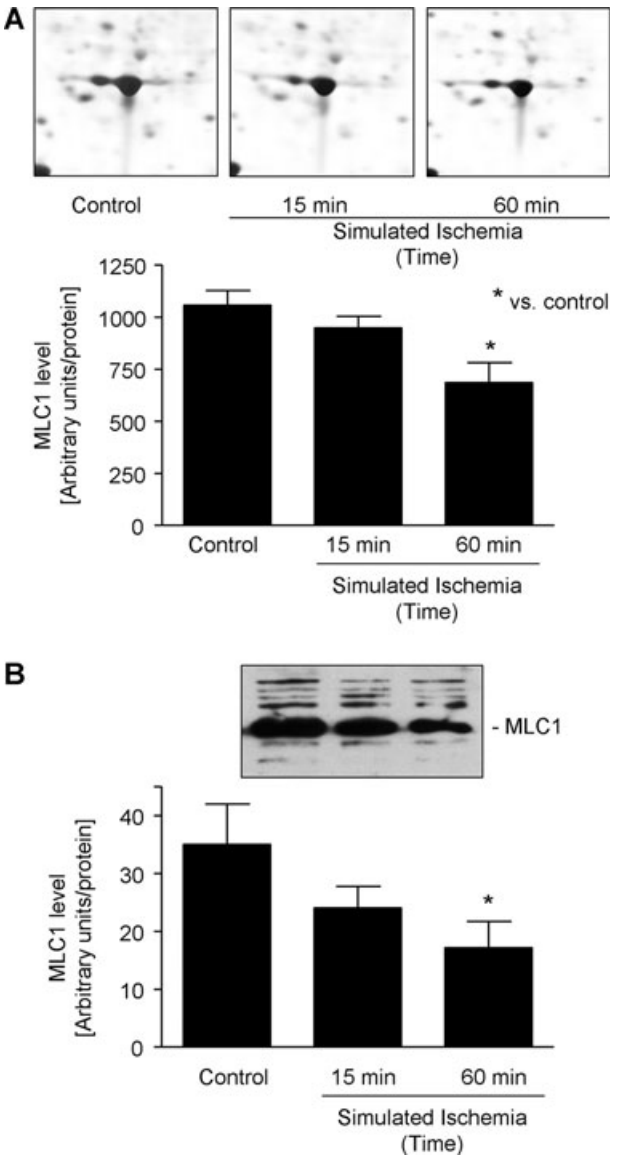


Fig. 3 MLC1 levels during 15 and 60 min. of simulated ischemia. (**A**) Upper panel shows representative enlarged 2D electrophoresis gel fragments of the MLC1 region from control, 15 and 60 min. ischemia groups. Lower panel shows the densitometric analysis of intensities of MLC1 protein spots. (**B**) Immunoblot analysis of MLC1 level in control group and those groups subjected to 15 and 60 min. of simulated ischemia. The insert shows a representative blot. Data are expressed as mean ± S.E.M., $n = 4$/group; $^*P < 0.05$.

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.

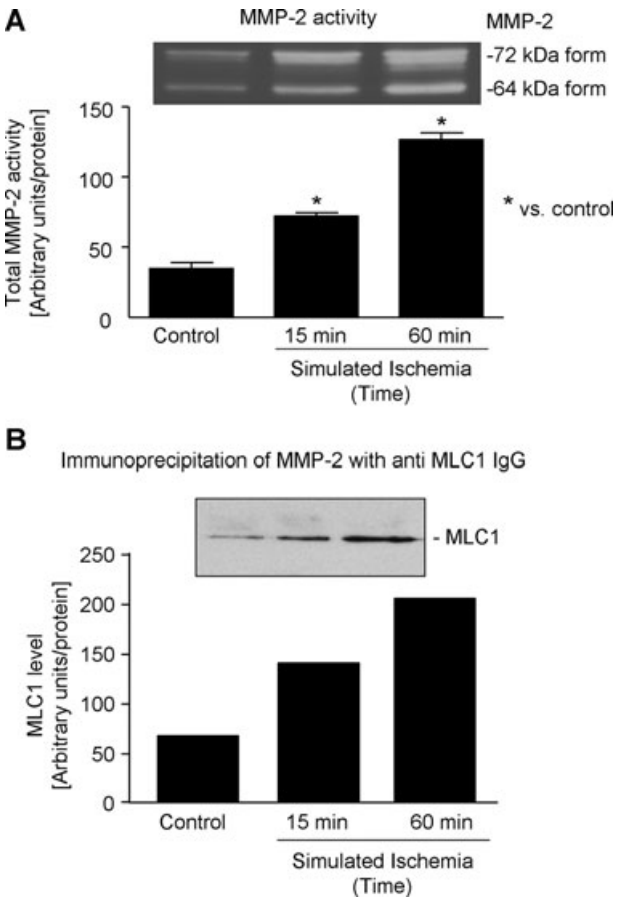


Fig. 4 MMP-2 activity and co-localization of MMP-2 and MLC1 in isolated rat cardiomyocytes subjected to simulated ischemia. (**A**) Analysis of total MMP-2 activity by zymography from control cardiomyocytes and those subjected to 15 and 60 min. of simulated ischemia. The insert shows a representative zymogram. Data are expressed as mean ± S.E.M., $n = 4$/group; $^*P < 0.05$. (**B**) Detection and assessment of MLC1 level co-localized with MMP-2 by immunoprecipitation.

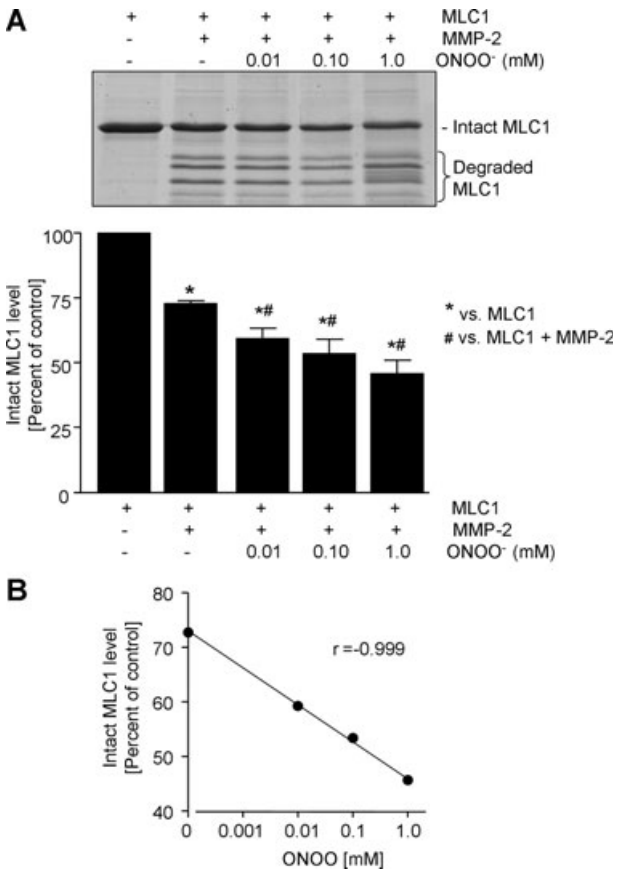


Fig. 5 The effect of $ONOO^-$ on i*n vitro* degradation of recombinant human cardiac MLC1 by MMP-2. (**A**) SDS-PAGE analysis of *in vitro* degradation of $ONOO^-$ treated MLC1 by MMP-2. The insert shows a representative SDS-PAGE. Data are expressed as mean ± S.E.M., $n = 4$/group; $^*P < 0.05$. (**B**) Correlation between intact MLC1 level and $ONOO^-$ concentration.

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).

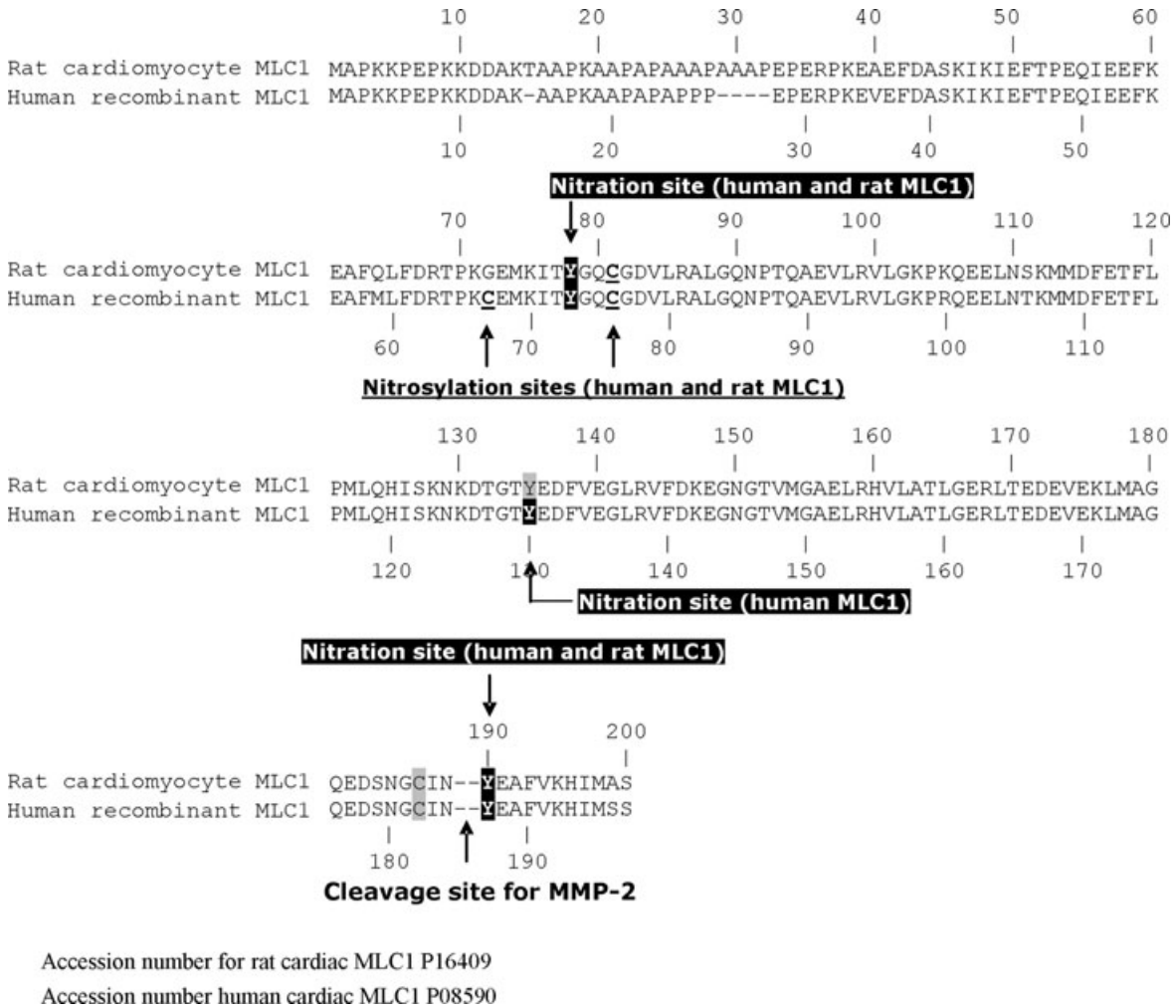
Effect of $ONOO^-$ on the degradation of recombinant human cardiac MLC1 by MMP-2 *in vitro*

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 nitration 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



Accession number for rat cardiac MLC1 P16409

Accession number human cardiac MLC1 P08590

Fig. 6 Mass spectrometry analysis for nitration and nitrosylation of human (P08590) and rat (P16409) cardiac MLC1. The nitrated amino acid residues are labelled in black boxes and nitrosylated amino acid residues are underlined. Both types of PTMs are indicated by the arrows. Non-modified tyrosine and cysteine residues are also shown and are highlighted in grey. The cleavage site for MMP-2 located between asparagine 189 (N189) and tyrosine 190 (Y190) is indicated.

protein. 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

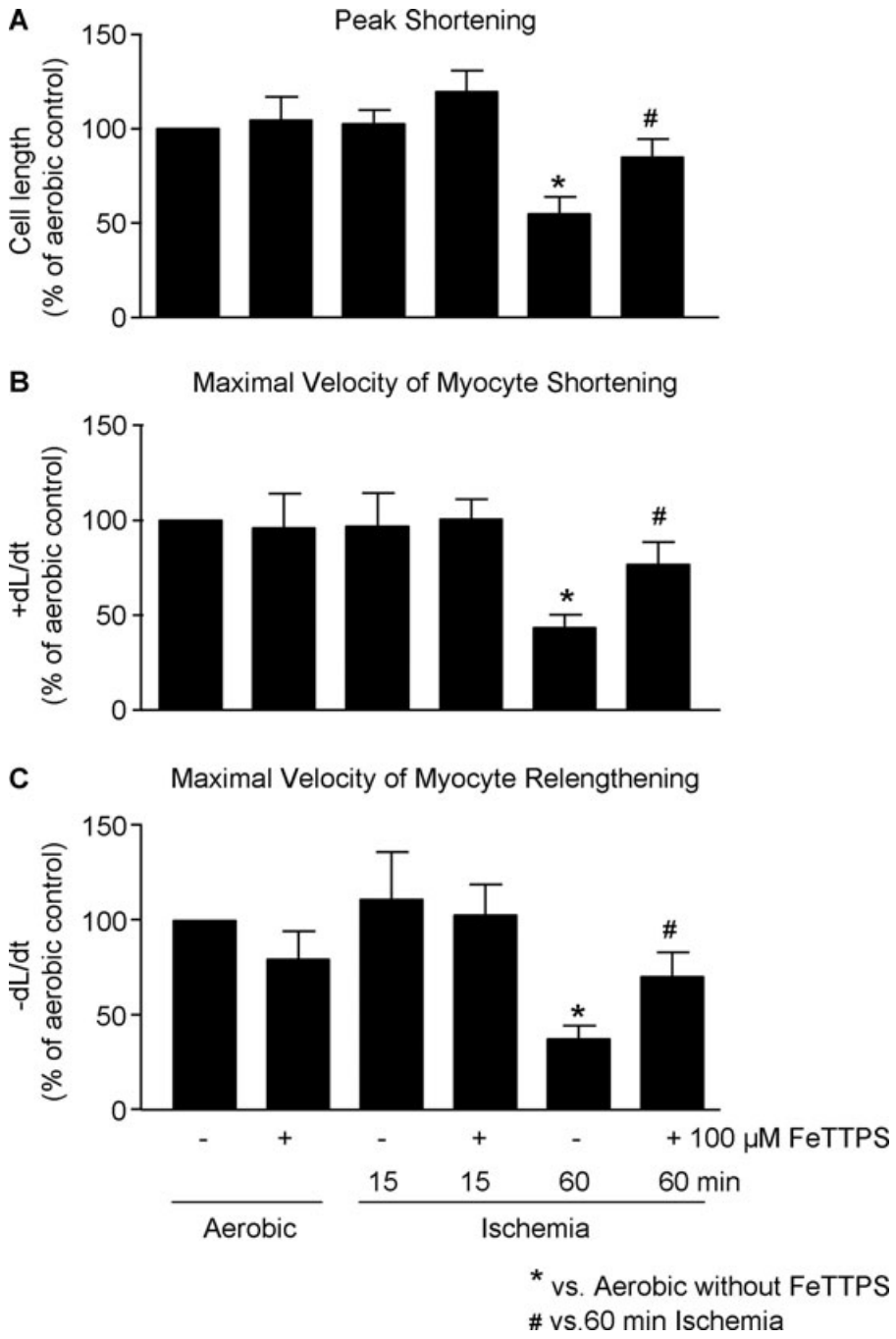


Fig. 7 Evaluation of contractile function of isolated rat cardiomyocytes subjected to simulated ischemia. Freshly isolated rat cardiacmyocytes were either maintained in aerobic conditions or subjected to 15 or 60 min. of simulated ischemia. In parallel, groups were pre-treated with 100 μM FeTPPS, a scavenger 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.

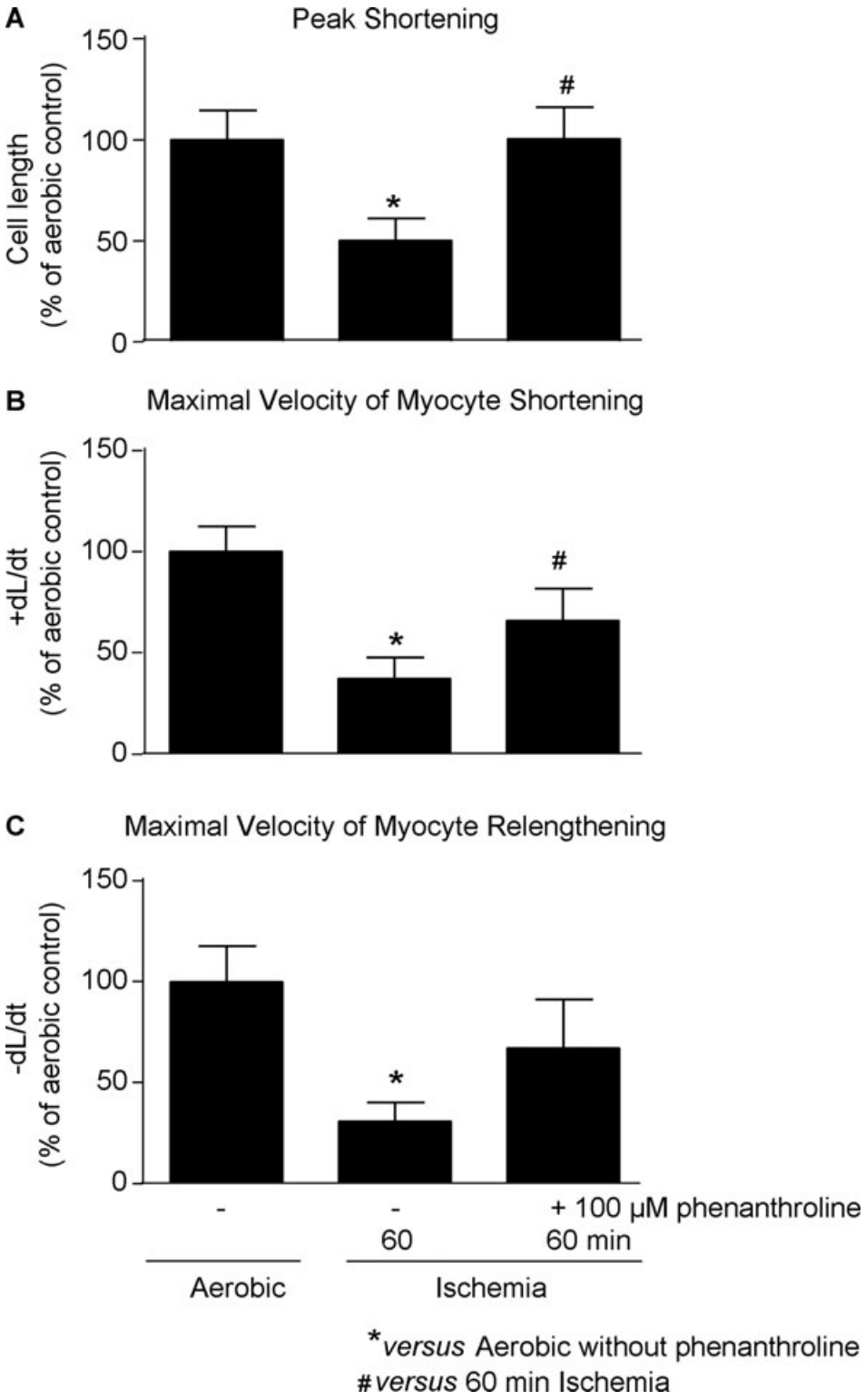


Fig. 8 Evaluation of contractile function of isolated rat cardiomyocytes subjected to simulated ischemia with phenanthroline treatment. Freshly isolated rat cardiacmyocytes were either maintained in aerobic conditions or subjected to 60 min. of simulated ischemia. In parallel, groups were pre-treated 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.

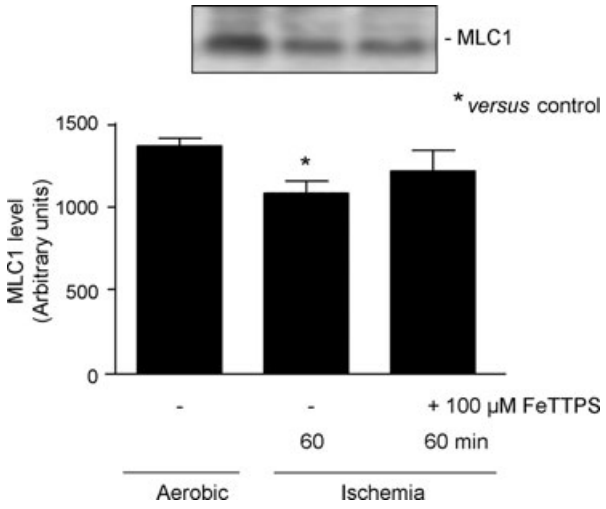


Fig. 9 Protection of MLC1 degradation by $ONOO^-$ scavenger FeTPPS. Cardiomyocytes subjected to 60 min. of ischemia in the presence or absence of FeTPPS pre-treatment were used for immunoblot analysis of MLC1 levels. Data are expressed as mean ± S.E.M., $n = 4$/group; $^*P < 0.05$ *versus* aerobic control.

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 μM of FeTPPS (Fig. 9) or with 100 μM 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

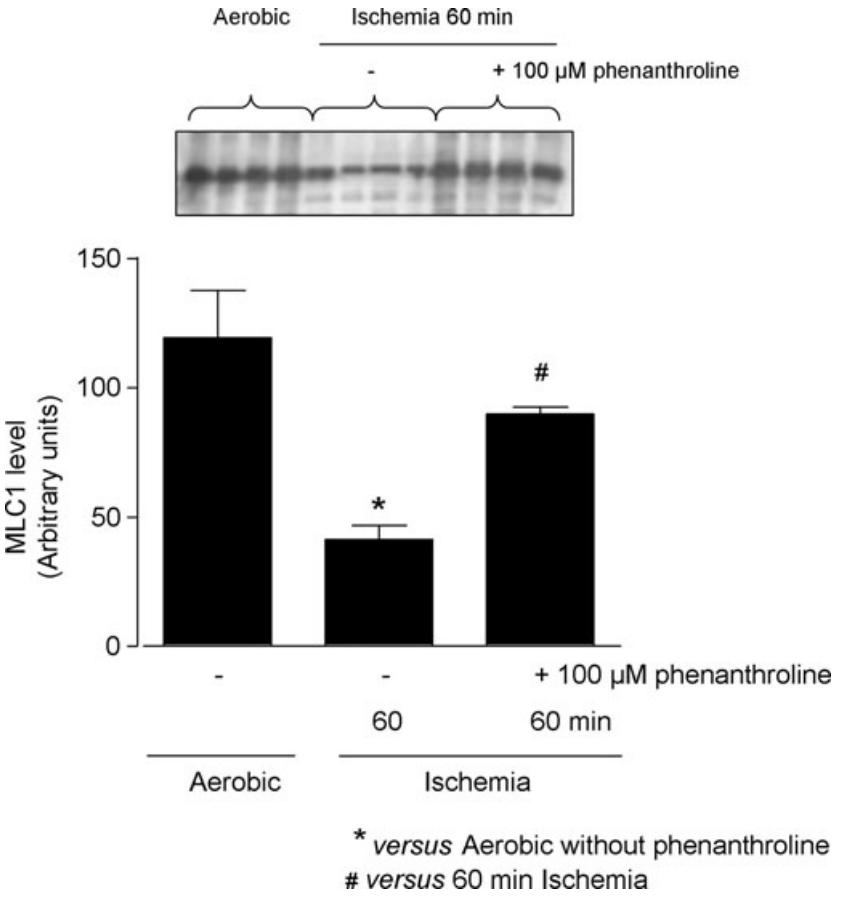


Fig. 10 Protection of MLC1 degradation by the MMP-2 inhibitor phenanthroline. Cardiomyocytes subjected to 60 min. of ischemia in the presence or absence of phenanthroline pre-treatment were used for immunoblot analysis of MLC1 levels. Data are expressed as mean ± S.E.M., $n = 4$/group; $^*P < 0.05$ *versus* aerobic control.

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 possible 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.

References

1. **Yellon DM, Hausenloy DJ.** Myocardial reperfusion injury. *N Engl J Med*. 2007; 357: 1121–35.
2. **Yasmin W, Strynadka KD, Schulz R.** Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc Res*. 1997; 33: 422–32.
3. **Zweier JL, Wang P, Kuppusamy P.** Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. *J Biol Chem*. 1995; 270: 304–7.
4. **Yao Z, Tong J, Tan X, *et al*.** Role of reactive oxygen species in acetylcholine-induced preconditioning in cardiomyocytes. *Am J Physiol*. 1999; 277: H2504–9.
5. **Schulz R, Wambolt R.** Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia-reperfusion injury. *Cardiovasc Res*. 1995; 30: 432–9.
6. **Lopez BL, Liu GL, Christopher TA, *et al*.** Peroxynitrite, the product of nitric oxide and superoxide, causes myocardial injury in the isolated perfused rat heart. *Coron Artery Dis*. 1997; 8: 149–53.
7. **Ferdinandy P, Schulz R.** Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *Br J Pharmacol*. 2003; 138: 532–43.
8. **Jugdutt BI.** Nitric oxide and cardioprotection during ischemia-reperfusion. *Heart Fail Rev*. 2002; 7: 391–405.
9. **Schulz R, Dodge KL, Lopaschuk GD, *et al*.** Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. *Am J Physiol*. 1997; 272: H1212–9.
10. **Digerness SB, Harris KD, Kirklin JW, *et al*.** Peroxynitrite irreversibly decreases diastolic and systolic function in cardiac muscle. *Free Radic Biol Med*. 1999; 27: 1386–92.
11. **Vescovo G, Ravara B, Dalla Libera L.** Skeletal muscle myofibrillar protein oxidation and exercise capacity in heart failure. *Basic Res Cardiol*. 2008; 103: 285–90.
12. **Hess DT, Matsumoto A, Kim SO, *et al*.** Protein S-nitrosylation: purview and parameters. *Nature reviews*. 2005; 6: 150–66.
13. **Kanski J, Behring A, Pelling J, *et al*.** Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: effects of

biological aging. *Am J Physiol*. 2005; 288: H371–81.

14. **Hong SJ, Gokulrangan G, Schoneich C.** Proteomic analysis of age dependent nitration of rat cardiac proteins by solution isoelectric focusing coupled to nanoHPLC tandem mass spectrometry. *Exp Gerontol*. 2007; 42: 639–51.
15. **Snook JH, Li J, Helmke BP, *et al*.** Peroxynitrite inhibits myofibrillar protein function in an *in vitro* assay of motility. *Free Radic Biol Med*. 2008; 44: 14–23.
16. **Van Eyk JE, Powers F, Law W, *et al*.** Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: identification of degradation products and effects on the pCa-force relation. *Circ Res*. 1998; 82: 261–71.
17. **Kanski J, Hong SJ, Schoneich C.** Proteomic analysis of protein nitration in aging skeletal muscle and identification of nitrotyrosine-containing sequences *in vivo* by nanoelectrospray ionization tandem mass spectrometry. *J Biol Chem*. 2005; 280: 24261–6.
18. **Sawicki G, Leon H, Sawicka J, *et al*.** Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. *Circulation*. 2005; 112: 544–52.
19. **Doroszko A, Polewicz D, Sawicka J, *et al*.** Cardiac dysfunction in an animal model of neonatal asphyxia is associated with increased degradation of MLC1 by MMP-2. *Basic Res Cardiol*. 2009; 104: 669–79.
20. **Wang W, Schulze CJ, Suarez-Pinzon WL, *et al*.** Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation*. 2002; 106: 1543–9.
21. **Wang W, Sawicki G, Schulz R.** Peroxynitrite-induced myocardial injury is mediated through matrix metalloproteinase-2. *Cardiovasc Res*. 2002; 53: 165–74.
22. **Spinale FG.** Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. *Physiol Rev*. 2007; 87: 1285–342.
23. **Chow AK, Cena J, Schulz R.** Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature. *Br J Pharmacol*. 2007; 152: 189–205.
24. **Schulz R.** Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. *Annu Rev Pharmacol Toxicol*. 2007; 47: 211–42.
25. **Gallagher G, Menzie S, Huang Y, *et al*.** Regional cardiac dysfunction is associated with specific alterations in inflammatory cytokines and matrix metalloproteinases after acute myocardial infarction in sheep. *Basic Res Cardiol*. 2007; 102: 63–72.
26. **Light P, Shimoni Y, Harbison S, *et al*.** Hypothyroidism decreases the ATP sensitivity of KATP channels from rat heart. *J Membr Biol*. 1998; 162: 217–23.
27. **Bouchard RA, Clark RB, Giles WR.** Role of sodium-calcium exchange in activation of contraction in rat ventricle. *J Physiol*. 1993; 472: 391–413.
28. **Watanabe T, Delbridge LM, Bustamante JO, *et al*.** Heterogeneity of the action potential in isolated rat ventricular myocytes and tissue. *Circ Res*. 1983; 52: 280–90.
29. **Diaz RJ, Wilson GJ.** Studying ischemic preconditioning in isolated cardiomyocyte models. *Cardiovasc Res*. 2006; 70: 286–96.
30. **Diaz RJ, Zobel C, Cho HC, *et al*.** Selective inhibition of inward rectifier K+ channels (Kir2.1 or Kir2.2) abolishes protection by ischemic preconditioning in rabbit ventricular cardiomyocytes. *Circ Res*. 2004; 95: 325–32.
31. **Diaz RJ, Losito VA, Mao GD, *et al*.** Chloride channel inhibition blocks the protection of ischemic preconditioning and hypo-osmotic stress in rabbit ventricular myocardium. *Circ Res*. 1999; 84: 763–75.
32. **Ferdinandy P, Danial H, Ambrus I, *et al*.** Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res*. 2000; 87: 241–7.
33. **Sawicki G, Jugdutt BI.** Detection of regional changes in protein levels in the *in vivo* canine model of acute heart failure following ischemia-reperfusion injury: functional proteomics studies. *Proteomics*. 2004; 4: 2195–202.
34. **Sawicki G, Dakour J, Morrish DW.** Functional proteomics of neurokinin B in the placenta indicates a novel role in regulating cytotrophoblast antioxidant defences. *Proteomics*. 2003; 3: 2044–51.
35. **Perkins DN, Pappin DJ, Creasy DM, *et al*.** Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 1999; 20: 3551–67.
36. **Cheung PY, Sawicki G, Wozniak M, *et al*.** Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation*. 2000; 101: 1833–9.
37. **Zhang WZ, Lang C, Kaye DM.** Determination of plasma free 3-nitrotyrosine and tyrosine by reversed-phase liquid chromatography with 4-fluoro-7-nitrobenzofurazan derivatization. *Biomed Chromatogr*. 2007; 21: 273–8.
38. **Mathews CE, Suarez-Pinzon WL, Baust JJ, *et al*.** Mechanisms underlying resistance of pancreatic islets from ALR/Lt mice to cytokine-induced destruction. *J Immunol*. 2005; 175: 1248–56.
39. **Hensley K, Maidt ML, Pye QN *et al*.** Quantitation of protein-bound 3-nitrotyrosine and 3,4-dihydroxyphenylalanine by high-performance liquid chromatography with electrochemical array detection. *Anal Biochem*. 1997; 251: 187–95.
40. **Laemmli UK.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227: 680–5.
41. **Fert-Bober J, Basran RS, Sawicka J, *et al*.** Effect of duration of ischemia on myocardial proteome in ischemia/reperfusion injury. *Proteomics*. 2008; 8: 2543–55.