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Co-treatment with conjugated linoleic acid and nitrite protects against myocardial infarction ☆

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ABSTRACT

According to the CDC, the most common type of heart disease is coronary artery disease, which commonly leads to myocardial infarction (MI). Therapeutic approaches to lessen the resulting cardiovascular injury associated with MI are limited. Recently, MicroRNAs (miRNAs) have been shown to act as negative regulators of gene expression by inhibiting mRNA translation and/or stimulating mRNA degradation. A single miRNA can modulate physiological or disease phenotypes by regulating whole functional systems. Importantly, miRNAs can regulate cardiac function, thereby modulating heart muscle contraction, heart growth and morphogenesis. MicroRNA-499 (miRNA-499) is a cardiac-specific miRNA that when elevated causes cardiomyocyte hypertrophy, in turn preventing cardiac dysfunction during MI. Previous studies revealed that combination treatment with conjugated linoleic acid (cLA) and nitrite preserved cardiovascular function in mice. Therefore, it was hypothesized that cLA and nitrite may regulate miRNA-499, thus providing cardiac protection during MI. To test this hypothesis, 12-week old mice were treated with cLA (10 mg/kg/d-via osmotic mini-pump) or cLA and nitrite (50 ppm-drinking water) 3 days prior to MI (ligation of the left anterior descending artery). Echocardiography and pressure–volume (PV)-loop analysis revealed that cLA and nitrite-treated MI mice had improved heart function (10 days following MI) compared to untreated MI mice. Treatment with cLA and nitrite significantly induced levels of miRNA-499 compared to untreated MI mice. In addition, treatment with cLA and nitrite abolished MI-induced protein expression of p53 and dynamin-related protein-1 (DRP-1). Moreover, the antioxidant enzyme expression of heme oxygenase-1 (HO-1) was elevated in MI mice treated with cLA and nitrite compared to untreated MI mice. Confocal imaging on heart tissue confirmed expression the levels of HO-1 and p53. Taken together, these results suggest that therapeutic treatment with cLA and nitrite may provide significant protection during MI through regulation of both cardiac specific miRNA-499 and upregulation of phase 2 antioxidant enzyme expression.

Introduction

Myocardial infarction (MI) affects over five million individuals in the US and remains a significant and yet unsolved health problem. Experimental MI in mice is an important disease model, in part due to the ability to study genetic manipulations. Additionally, surgical induction of MI allows precise timing and location of a coronary event [1]. MI results in an insufficient blood supply to cardiac tissue, which leads to myocardial inflammation followed by scar formation at the site of infarction, as well as changes in the non-infarcted myocardium [2–4].

MI is characterized by significant changes in gene expression, many of which represent adaptive or maladaptive responses to stress [5–9]. The resulting cardiac stress induces rapid changes in gene expression immediately following MI [10]. Cardiomyocyte cell death is a consequence of myocardial injury, which occurs as early as the initiation of acute MI [11]. Cardiomyocyte death or apoptosis is a key factor in transition from cardiac hypertrophy to heart failure [12]. p53 is a well-known transcription factor which mediates apoptosis by activating the manifestation of pro-apoptotic genes [13]. Previous studies have demonstrated that p53 activity is enhanced during MI and that p53 plays a critical role in the regulation of hypoxia-induced apoptosis of cardiomyocytes [14,15]. Thus, p53 becomes an important therapeutic target, with regard to attenuation of cardiac apoptosis and consequent heart failure.

MicroRNAs quantitatively regulate mature-RNAs, which affect the cardiac transcriptional output and cardiac function [16]. MicroRNAs (miRNAs) are endogenous, single-stranded non-coding RNAs ranging 18 to 24 nucleotides in length, that play an important role

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in gene regulation [16,17]. In screening for miRNAs enriched in the heart, we found an abundant miRNA, miRNA-499, which is an evolutionarily conserved muscle-specific microRNA that is encoded within the intron of myosin heavy chain and is highly expressed in the cardiac ventricles [16–18]. Plasma miRNA-499 is known as a biomarker of acute MI, as plasma miR-499 has been observed in individuals with MI [19].

Studies have shown that diets with diversified fats may be an effective strategy in decreasing risk of cardiovascular disease (CVD) [20,21]. Conjugated linoleic acid (cLA) is a fatty acid that occurs as a mixture of positional and geometric isomers of linoleic acid (LA), which is produced in ruminant animals via an enzymatic isomerase reaction [22]. cLA contains 18 carbon atoms with two conjugated double bonds separated by a single bond [23]. cLA is found naturally in food products from these animals predominantly as the cis-9,trans-11 form, whereas synthetic cLA preparations consist of a few different isomers with an approximately equal amount of cis-9,trans-11 and trans-10,cis-12 cLA [21,24]. Select modified fatty acids, such as nitrated fatty acids are known to induce pleuripotent anti-inflammatory effects [25]. Recent literature suggests that dietary cLA and nitrite supplementation in rodents elevates NO_2-cLA levels in the plasma and tissues, inducing heme oxygenase-1 (HO-1) expression in the target tissue [26]. HO-1 catalyzes the oxidation of heme—producing biliverdin, iron, and carbon monoxide (CO) [27,28]. Importantly, biliveridin is converted to bilirubin, a known potent antioxidant. Additionally, iron can be sequestered by ferritin, leading to off target anti-apoptotic effects and carbon monoxide has similar characteristics to nitric oxide, facilitating numerous biological functions including anti-inflammatory effects [28–30].

Ultimately, heart function is highly dependent on ATP generation. The heart is enriched with mitochondria that provide the energy required for cardiomyocyte function [31]. Therefore, it is known that mitochondria play a critical role in development and progression of many cardiac diseases such as hypertrophy and myocardial infarction. Previous studies suggest that mitochondria are highly dynamic and that changes in mitochondrial shape can affect a variety of biological processes such as apoptosis and fibrosis [32,33]. Mitochondria are dynamic organelles, which constantly undergo fission and fusion [32–34]. These two opposing processes are regulated by the mitochondrial fission proteins mitofusin and the mitochondrial fission proteins Drp-1 [31]. During apoptosis, Drp-1 foci accumulates on mitochondria and can enhance mitochondrial fission [35,36].

In the present study an in vivo model of MI is used to further elucidate the possible mechanism whereby combination treatment with cLA and nitrite is cardioprotective. Herein we demonstrate that cLA and nitrite significantly induced levels of miR-499 in heart compared to untreated MI mice. Additionally, co-treatment significantly reduced levels of p53 expression and induced expression of HO-1. These results suggest that treatment with cLA and nitrite may provide significant protection during MI through regulation of cardiac specific miRNA and upregulation of phase 2 antioxidant enzyme expression.

Materials and methods

Animals

Mice were fed standard chow and water ad libitum. All animal procedures were reviewed and approved by an independent Institutional Animal Care and Use Committee of the University of Louisville, School of Medicine. In addition, all studies were performed in accordance with animal care and use guidelines of the National Institutes of Health.

Mouse model of myocardial infarction

Male C57BL/6 mice, 10- to 12-wks old, were anesthetized with isoflurane, intubated and ventilated with CWE advanced ventilator (Webster, TX). Body temperature was maintained with an Indus Temperature feedback/surgical table and ECG system. Aseptic procedure was used for preparation of the surgical site through scrubbing with a 0.8% chlorhexidine solution. A left thoracotomy was performed via the fourth intercostal space and the lungs retracted to expose the heart. After opening the pericardium, the left anterior descending (LAD) coronary artery was ligated with an 8–0 silk suture near its origin between the pulmonary outflow tract and the edge of the atrium. Ligation was deemed successful when the anterior wall of the left ventricle (LV) turned pale. The lungs were inflated by increasing positive end-expiratory pressure, and the thoracotomy side was closed in layers. The lungs were re-expanded, and the chest was closed. The animals were removed from the ventilator and allowed to recover on a heating pad. Mice were checked daily for signs of pain or distress and buprenex at 0.05 mg/kg SQ is given before and every 12 h for 48 h. Mice were treated with cLA (10 mg/kg/d-via osmotic mini-pump) or cLA and nitrite (50 ppm-drinking water) 3 days prior to ligation of the LAD artery.

In vivo hemodynamic measurements

Isoflurane was used to anesthetize the mouse and conductance readings were made for ~15–20 min, prior to harvesting heart tissue. Briefly, the mouse was placed in a supine position on a 37 °C pad under the surgical microscope and the limbs were restrained with tape. A 0.5 cm skin incision was performed in the right neck area and the carotid artery was isolated using silk sutures. The cranial aspect of the carotid artery was ligated and a microsurgical clip was placed on the proximal carotid artery for hemostasis. An arteriotomy was performed with microsurgical scissors, and a 1.2 French conductance catheter (Transonic, London, ON, Canada) was introduced into the carotid artery and advanced retrograde across the aortic valve into the left ventricle. The catheter was advanced under continuous hemodynamic monitoring to issue proper placement in the left ventricle (LV). The catheter was secured within the carotid artery with the proximal suture. LV pressure–volume loops were recorded in the steady state. Loops were recorded using the iWorks data acquisition software package, and analyzed using the LabScribe2 pressure–volume data analysis software package (iWorks, St. Albans, Vermont).

Real-time quantitative PCR

Total heart tissue was homogenized in TRIzol and RNA was isolated, as previously described [37]. Complimentary DNA was synthesized using oligo (dT) primers (Promega Corporation, Madison, WI) and stored at −80 °C until experiments were performed. Reactions were done using 2000 ng/μL cDNA and SYBR Green Master Mix (BioRad Laboratories, Hercules, CA). BioRad CFX Manager (BioRad Laboratories, Hercules, CA) software was used to analyze results. Gene expression was normalized with mRNA expression of 18S. Samples were analyzed in triplicate using $N=3$ for each independent experiment. The following primer sequences were used:

murine pre-miR-499
Forward-1: 5′-GGGCAGCTGTTAAGACTTGC-3′
Forward-2: 5′-TGGCTTTCTGCAGGCTGC-3′
Reverse: 5′-AGGCAGCAGCACAGACTTG-3′
murine miR-499
Forward: 5′-GGG-TGG-GCA-GCT-GTT-AAG-AC-3′
Reverse: 5′-AGG-CAG-CAG-CAC-AGA-CTT-G-3′

murine DRP-1
Forward: 5′-AAA CTC CTA TCA CGC TCA TCA-3′
Reverse: 5′-CTC ATC CTC CAC GCA TCC T-3′

Western blot analysis

Heart tissue homogenate (100 μg) was electrophoresed using SDS-PAGE method as previously described [38,39]. Affinity purified GAPDH (1:3000) (Trevigen, Gaithersburg, MD) p53 (1:1000) (Calbiochem, Billerica, MA) and HO-1 (1:1000) (Stressgen Biotechnologies, Farmingdale, NY) antibodies were detected using specie-appropriate horse radish peroxidase-labeled secondary antibodies.

Histology and confocal microscopy

Hearts were collected from the mice and thoroughly washed in PBS. Using Peel-A-Way disposable plastic tissue embedding molds (Polysciense Inc, Washington, PA) filled with tissue freezing media (Triangle Biomedical Sciences, Durham, NC), hearts were preserved and stored at −80 °C until analysis. Tissue sections (5 μm in thickness) were made using Leica CM 1850 Cryocut microtome (Bannockburn, IL, USA). Sections were placed on super frost plus glass slides, air-dried, and processed for Immunohistochemistry (IHC) staining.

Immunohistochemistry

Immunohistochemistry and laser-scanning confocal microscopy were used to visualize MI-induced changes in p53 and HO-1 expression and localization. Immunohistochemistry was performed on frozen tissue sections using a standard IHC protocol. Primary antibodies were applied overnight (anti-p53, Calbiochem, Billerica, MA and anti HO-1, Stressgen Biotechnologies, Farmingdale, NY). Secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Carlsbad, CA) were applied for protein immunodetection. Stained slides were analyzed for fluorescence using a laser scanning confocal microscope (Olympus FluoView-1000, objective 40X) set at the appropriate filter settings. The total fluorescence (green or red) intensity in 5 random fields (for each experimental sample) was measured with image analysis software (Image-Pro Plus, Media Cybernetics). Fluorescence intensity values for each experimental group were averaged and presented as fluorescent intensity units (FIU).

Statistical analysis

Values are presented as mean ± SD. Differences between groups were tested by one-way ANOVA and Bonferroni's multiple comparison post hoc analysis. Data was considered statistically significant for $p < 0.05$.

Results

Co-treatment of cLA and nitrite increases heart weight following MI

Hearts isolated from MI (10 days) and treated mice were found to be enlarged, with ventricular dilatation (Fig. 1A). The heart weight/tibia length ratio was significantly increased in MI and single treated mice compare to control hearts, where co-administration cLA and nitrite was significantly higher than MI hearts with or without single treatment (Fig. 1A). There was no significant difference in body weight/tibia length ratio in all the group of mice (Fig. 1B).

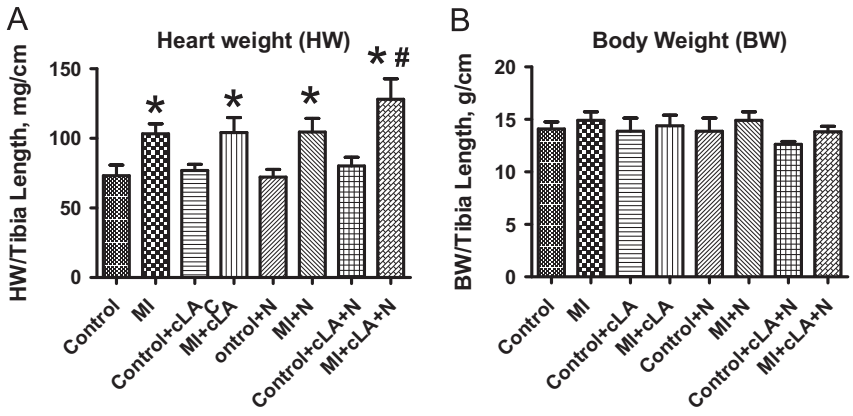

Fig. 1. Co-treatment with cLA and nitrite increases heart weight following MI. Heart weight is significantly increased following cLA or nitrite with MI, or in MI alone (A). cLA and nitrite co-treatment results in a further increase in heart weight, which is significant compared to MI alone (A) ($^{*}p < 0.05$ to control), $^{\#}p < 0.05$ to MI, $n=8$), where overall body weight is unchanged (B).

Table 1
Hemodynamics of MI and control mice based on pressure–volume. All values are expressed as mean ± S.D. ESP, end-systolic pressure; EDP, end-diastolic pressure; SV, stoke volume; EF, ejection fraction; CO, cardiac output; BPM, beats per minute; Significance is designated as follows: $^{*}p < 0.05$, compared to control, $^{\#}p < 0.05$ compared to MI and $^{*\#}p < 0.05$ compared to control and MI, $^{\wedge}p < 0.05$ compared to MI+cLA+N, where $n=6$ for each group.

	HR BPM	ESP mmHg	EDP mmHg	ESV μL	EDV μL	SV μL	CO μL/min	EF %
Control	552.00 ± 1.79	80.88 ± 0.49	6.95 ± 0.67	15.43 ± 0.37	40.66 ± 1.72	25.23 ± 1.53	13926.93 ± 449.70	62.05 ± 2.13
MI	555.78 ± 8.67	78.36 ± 3.72	2.73 ± 1.25^	55.43 ± 3.72*	72.77 ± 2.40*	17.66 ± 3.72*	9800.19 ± 1218.05*	23.00 ± 4.17*
MI+cLA	546.82 ± 4.30	79.80 ± 9.78	2.83 ± 2.55^	60.90 ± 2.34*	75.40 ± 0.47*	14.51 ± 2.19*	7644.82 ± 1008.05*	19.66 ± 1.88*
MI+N	538.98 ± 12.14	75.75 ± 0.85	3.77 ± 0.10	59.21 ± 4.57*	75.46 ± 3.89*	16.23 ± 1.93*	8608.74 ± 1898.04*	21.35 ± 2.46*
MI+cLA+N	556.82 ± 4.30	85.79 ± 0.75	10.26 ± 3.26	30.90 ± 2.34*#	56.90 ± 2.34*#	26.56 ± 2.36#	14767.36 ± 1121.31#	45.42 ± 2.13*#

cLA and nitrite co-treatment improves cardiac function after MI

MI is characterized by ventricular chamber dilation and dysfunction (Table 1). We observed a significant rightward shift of the PV loop in MI and single treated mice (Fig. 2). Chamber dilation also resulted in significant increase in both end-systolic and end-diastolic volume (Fig. 2, Table 1). As shown in Fig. 2, invasive hemodynamic measurements showed a reduction in EF in MI vs. the control groups (Fig. 2, Table 1). Treatment did not change EF in control animals (Fig. 2, Table 1). However, co-administration with cLA and nitrite shifted PV-loop to the left compared to MI PV-loop, indicating that the overall heart function is improved in cLA and nitrite-treated mice following MI.

Mitochondrial fission factor DRP-1 is significantly increased in mice following MI

MI caused a robust increase in Drp-1 expression in MI (Fig. 3). Individual treatments with cLA or nitrite increased expression of Drp-1 compared to control mice, but the expression was overall lower than with MI-induction (Fig. 3). Co-treatment with cLA and nitrite attenuated Drp-1 expression in MI mice (Fig. 3).

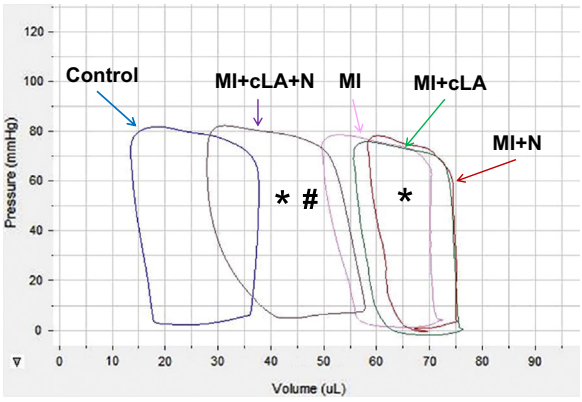

Fig. 2. Co-treatment with cLA and nitrite significantly improves cardiac function after MI. Treatment with cLA exacerbates cardiac injury after MI, where combination treatment (supplementation with nitrite) rescues heart function following MI ($^{*}p<0.05$ to control, $^{\#}p<0.05$ to MI, $n=8$).

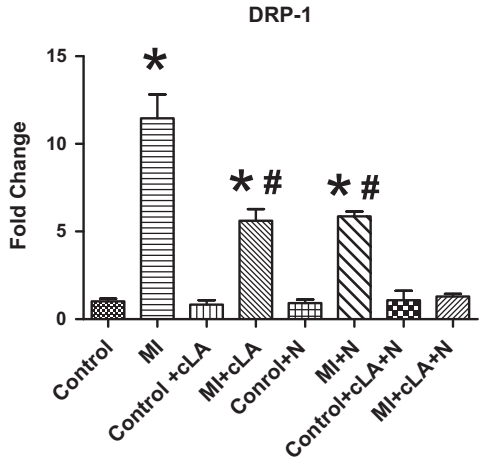

Fig. 3. Mitochondrial fission factor DRP-1 is attenuated in mice treated with cLA and nitrite following MI. Nitrite- or cLA-treated MI mice have decreased levels of DRP-1 compared to MI alone. Combination treatment with cLA and nitrite significantly lowers the level of Drp-1 in MI mice, comparable to control values ($^{*}p<0.05$ to control, $^{\#}p<0.05$ to MI).

Co-treatment of cLA and nitrite regulates pre miRNA-499 and miRNA-499 during MI

To investigate the specific role of miRNA-499 in regulation of cardiomyocyte hypertrophy during MI, miRNA-499 levels were measured in the heart (Fig. 4). Both pre-miRNA-499 and miRNA-499 are increased following MI with combination treatment of cLA and nitrite (Fig. 4). There were no significant differences in single treated MI mice or with MI alone (Fig. 4).

cLA and nitrite treatment attenuates p53 expression following MI

Expression of p53 was defined by immunohistochemical staining of cryo-sectioned hearts (Fig. 5A). To support the immunohistochemical results that miRNA-499 blocks p53 after co-administration treatment with cLA and nitrite during MI, the level of p53 was assessed by Western blot analysis (Fig. 5). The protein level of p53 was increased following MI and in single treated mice, but was abolished after co-treatment with cLA and nitrite (Fig. 5B and D). The p53 staining support the data obtained via Western blot analysis.

HO-1 expression increases in MI following cLA and nitrite treatment

HO-1 expression is increased, as demonstrated using immunohistochemical staining of cryo-sectioned heart tissue (Fig. 5A). To support the hypothesis that co-treatment with cLA and nitrite during MI induces HO-1 level, the expression of HO-1 was assessed using Western blot analysis (Fig. 5). Following treatment with cLA and nitrite, HO-1 protein expression is significantly increased (Fig. 5C and D).

Discussion

This study examines the effects of cLA with and without nitrite supplementation on cardiovascular injury following MI. Considering the initiative to replace saturated fats with so-called 'healthy fats', to promote overall weight loss and maintenance, the health effects of fats such as cLA require investigation. The principal findings demonstrated here, are that cLA is not protective in MI, but instead worsens cardiac injury. However, supplementation with nitrite in cLA-(10 mg/kg/d-via osmotic mini-pump) treated mice, leads to cardioprotection in MI injury. Specifically, cLA and nitrite co-treatment significantly increases miRNA499 and blocks mitochondrial fission through inhibition of p53. Further, co-treatment with cLA and nitrite also induces HO-1 protein expression, which supports cardioprotection in this MI model. Overall, cLA and nitrite mediate protection and result in improved heart function in this murine model of MI.

It is known that mitochondria undergo fission and fusion events continuously in non-disease states [40]. Mitochondrial morphological dynamics affect the outcome of ischemic heart damage and pathogenesis [40]. The Drp1 protein plays an important role in fission, regulating mitochondrial membrane dynamics [34,40,41]. Drp1 exists as small oligomers, located primarily at the mitochondrial outer membrane. These oligomers can connect to each other, forming larger multimeric structures, thus mediating mitochondrial division [5,34,41,42]. In previous studies Drp1 has been identified as a mediator of mitochondrial morphological changes and cell death during cardiac ischemic injury [42]. It has been reported that miR-499 affects DRP-1 mediated apoptosis and the severity of MI and cardiac dysfunction during heart disease [18]. Our data suggest that MI significantly increases Drp1 expression in the heart of cLA- and nitrite-treated, as well as in non-treated mice (Fig. 3). Drp1

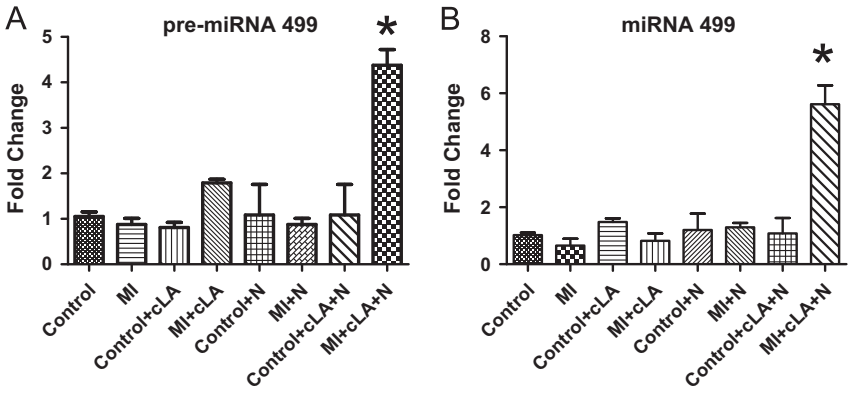

Fig. 4. Co-treatment with cLA and nitrite increases pre-miRNA-499 and miRNA-499 during MI. Pre-miRNA-499 (A) and miRNA-499 (B) is increased in MI mice after combination treatment with cLA and nitrite (*$p < 0.05$).

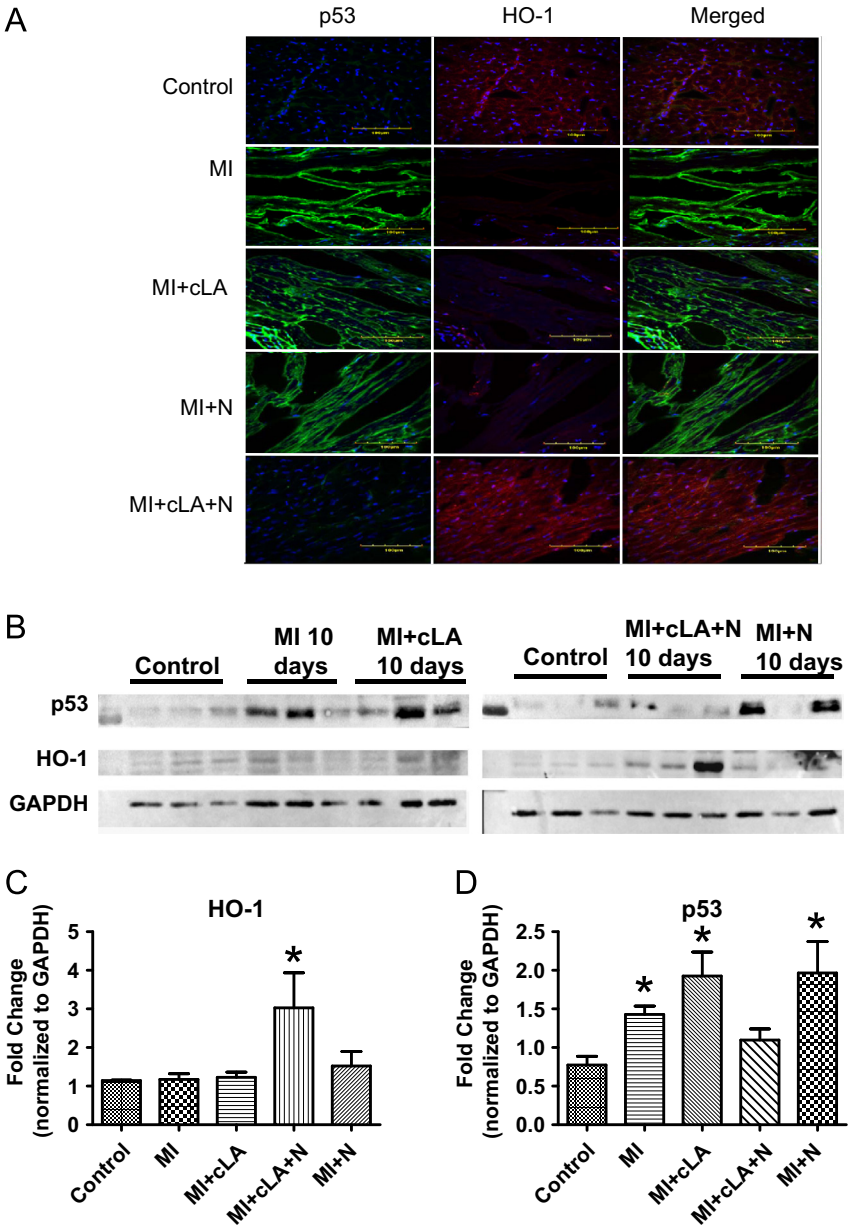

Fig. 5. Co-treatment with cLA and nitrite attenuates p53 and induces HO-1 expression following MI. Confocal microscopy (A): heart tissue was labeled with p53 (green), HO-1 (red), and cell nuclei was labeled with DAPI (blue). Western blot analysis reveals that p53 is significantly increased in cardiac tissue following MI (B). Quantitated protein expression reveals that co-administration of cLA and nitrite lowers p53 levels, while increasing expression of HO-1 (C) and (D) (*$p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression was abolished following co-treatment with cLA and nitrite in MI mice.

There is evidence that miRNA-499 controls the apoptotic pathway thorough regulation of p53 [18]. Our data supports the hypothesis that miRNA-499 decreases Drp1 levels after combination treatment with cLA and nitrite. Myocardial infarction is known to cause cardiomyocyte ischemia, in turn, leading to p53-dependent cardiomyocyte apoptosis [14,43]. The current study extends previous findings by demonstrating that ischemia causes increased expression of p53 following MI in non-treated and cLA- or nitrite-treated mice and provides significant implications with regard to the molecular mechanism of cardiomyocyte apoptosis following myocardial infarction.

Previous studies have shown that pharmacological delivery of nitrated fatty acids lead to protection of cardiovascular injury [44,45]. These studies have focused on protective signaling pathways including inhibition of p65 subunit of NFκB and increased expression of HO-1. In a model of stenosis femoral artery injury, nitrated oleic acid induced vascular expression of HO-1, mediating protection against neointimal hyperplasia [45]. Nitrated oleic acid inhibited activation of NFκB in a murine model of ischemia reperfusion injury, resulting in reduction of infarct size [44]. More recent data suggests that dietary cLA is a privileged substrate for nitration reactions facilitated by mitochondria, digestion, and macrophage activation and following metabolic stress such as MI [26]. Co-administration of dietary cLA and nitrite supplementation raises NO_2_cLA levels in plasma and tissues, which in turn induces HO-1 expression in target organs [26].

In the failing heart, HO-1 has anti-apoptotic effects by attenuating cell loss, p53 expression, and pathological heart remodeling [30]. Cytoprotective effects of HO-1 are mediated by reaction products CO and biliverdin. Importantly, biliveridin is converted to bilirubin, a known potent antioxidant [30,46]. Previous studies have shown induction of HO-1 to result in cardiac protection during ischemia/reperfusion injury [30,47–49]. Our results establish that the upregulation of HO-1 during co-treatment with cLA and nitrite in MI, ameliorates cardiac dysfunction. Further, sustained HO-1 expression in MI mice treatment with cLA and nitrite promotes miRNA-499 expression and limits Drp1and p53 expression.

Overall, these data reveal links among p53, HO-1, miR-499, and Drp1 with regard to regulation of the apoptotic program programed cell death in the heart. Taken together, these results suggest that therapeutic treatment with cLA and nitrite may provide cardiac protection during MI through the regulation of both cardiac specific miR-499, as well as induction of cardiac HO-1 expression. Further, this data suggests that modulation of miR-499 may represent a therapeutic approach to treat apoptosis-related cardiac disease, including MI.

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