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Nitrite Treatment Rescues Cardiac Dysfunction in Aged Mice Treated with Conjugated Linoleic Acid

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Abstract

Conjugated linoleic acid (cLA) is a commercially available weight loss supplement that is not currently regulated by the FDA. Numerous studies suggest that cLA mediates protection in diseases including cancer, diabetes, atherosclerosis, immune function, and obesity. Based upon these reports, it was hypothesized that supplementation of cLA would improve heart function in aged wild-type (WT) mice. At 10 months of age, mice were treated with cLA, nitrite, or the combination of the two. Echocardiograms revealed that cardiac function was decreased in aged compared to young WT mice, as determined by percent of fractional shortening (%FS). Also, contrary to the hypothesis, mice that received cLA (6-week treatment) had significantly worse cardiac function compared to controls. This effect was attenuated when mice were co-treated with cLA and nitrite. Taken together, these results suggest that cLA-mediated cardiac injury can be circumvented following nitrite supplementation in a murine model of aging.

Keywords

conjugated linoleic acid; nitrite; eNOS; heart function

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Introduction

Conjugated Linoleic Acid (cLA) refers to a group of naturally occurring isomers of linoleic acid (LA) characterized by conjugated double bonds, which may occur at several different positions [1]. There are two prominent isomers, where the cis-9, trans-11 isomer is naturally present in ruminant meat and dairy products [2], while trans-10, cis-12 occurs in partially hydrogenated oils [3]. Both isomers are also synthetically derived. cLA is sold commercially as a dietary supplement, and is not currently regulated by the FDA. Supplements are often a mixture of various isomers and it is important to note that isomers do not always behave uniformly because isomer specific effects are possible.

Commercial cLA is commonly used as a weight loss supplement and the mechanism through which cLA promotes weight loss has been speculated. Reports suggests that cLA induces weight loss in numerous manners such as reducing adiposity through an increase in thermogenesis, alterations in mitochondrial activity, and modulation of energy expenditure [4]. Furthermore, although both isomers have been implicated in weight loss, t10, c12 is known to reduce adiposity, as c9, t10 simply lessens body weight gain. However, recent studies have shown that t10, c12 can increase that expression of leptin whereas c9, t11 has no effect on leptin expression [4]. Notably, neither isomer had an effect on food intake [4].

cLA is reported to have favorable effects in various disease states, including atherosclerosis. Specifically, the c9, t11 isomer may be the main isomer involved in reducing atherosclerosis development, indicated by a reduction in plasma cholesterol, glucose and lesion area [5]. It has been established that cLA is a peroxisome proliferator activated receptor (PPAR) agonist. Importantly, PPARs have various effects on the events that lead to the development of atherosclerosis [6], suggesting that cLA stimulated PPAR could be one mode of atherosclerosis reduction. cLA has also been studied with regard to immune function, where during an acute phase or inflammatory immune response, cLA can alter immune function through decreased production of eicosanoids as well as suppression of pro-inflammatory cytokines [7]. However, other reports suggest that cLA has no effect on immune function in healthy individuals [7].

While there are numerous studies demonstrating the possible health benefits of dietary supplementation with cLA, it is essential to mention the possible adverse effects. For instance dietary supplementation of cLA has been shown to increase inflammatory markers in white adipose tissue such as MCP-1, as well as increasing hepatic steatosis [4]. cLA has also been shown to inhibit angiogenesis in both atherosclerosis and tumor growth, slowing the progression of disease [8]. However, angiogenesis is essential for endothelial function, so inhibition may be detrimental to overall cardiovascular health.

Inflammatory vascular diseases are often marked with a loss of NO bioavailability. This loss could be attributed to many events such as, uncoupling of endothelial nitric oxide synthase (eNOS), which results in an increase in reactive oxygen species (ROS) and a lower production of NO. Reduced eNOS can result in increased blood pressure, increased leukocyte adherence, and platelet aggregation [9]. Many of these studies reinforce the importance of maintaining physiological NO levels. NO is expressed throughout the

vasculature and is essential for cardiac function. In addition to being responsible for regulating vascular tone and angiogenesis, expression of eNOS has been shown to provide protection against an array of diseases. This protection is often offered through the release of a basal level of NO. Importantly, L-arginine produced NO is dependent on oxygen availability, whereas NO produced via nitrite-nitrate occurs as oxygen decreases, indicating a possible secondary system to ensure NO production [10].

While eNOS is important for NO bioavailability in the vasculature, it is not the only enzyme which contributes to maintaining vascular homeostasis. One such family of enzymes are NADPH oxidases. The expression of NADPH oxidases varies between disease states and it is generally accepted that under physiologic conditions, vascular NADPH oxidases have a relatively low level of constitutive activity [11]. However, enzyme activity can be increased in response to stimuli [11]. Nox2, one member of the NADPH oxidase family, is the most widely expressed isoform in vasculature. Importantly, Nox2 activation affects NO bioavailability [11].

Recent studies suggest that the supplementation of inorganic nitrite can counteract adverse effects caused by a lack NO bioavailability due to aging. NO produced by inorganic nitrite supplementation is rapidly absorbed from the circulation by peripheral tissues and stored in cells until conversion to NO is needed [12]. Also, it has been demonstrated that nitrite can be reduced to NO in a biological system, mainly under acidic conditions [13]. Importantly, inorganic nitrite has been “Generally Recognized as Safe” by the FDA and is well-tolerated with little or no side effects at non-toxic doses [12]. The aforementioned studies indicate that inorganic nitrite supplementation is a viable option for replacing NO that is lost.

Numerous studies have demonstrated that with aging comes a loss of physiological NO. Studies have shown that supplementing with inorganic nitrite increases circulating tissue nitrite bioavailability in old mice [12]. Furthermore, it was established in old mice that nitrite restored endothelial NO bioavailability, as well as endothelial function [12]. Moreover, it has been shown that eNOS can use nitrite as a substrate, restoring NO production to baseline levels [13].

Aging results in an increased risk for cardiovascular disease and subsequent cardiac dysfunction. Many studies focus on therapeutic intervention and cardiac preservation. Previous literature demonstrates that cardiac function is diminished in an aged model due to both structural and functional changes. Moreover, these changes can affect the modulation of cardiovascular diseases [14]. Due to the numerous reports of cLA's beneficial applications in multiple diseases, it was hypothesized that it would attenuate the cardiac dysfunction that accompanies aging. Thus, the focus of this study was to investigate the effect of supplementation with c9, t11 cLA on cardiac function and subsequent physiological signaling in an aged mouse model.

Methods

Animals and experimental design

All animal studies were approved by the University of Louisville Institutional Animal Care and Use Committee. Adult C57BL/6J mice were obtained from Jackson Laboratories at 8-10 weeks of age and aged to 38 weeks prior to administration of treatment. Weights of mice were recorded three times per week throughout the duration of the experiment. Animals were divided into four groups; control, conjugated linoleic acid (cLA), nitrite, and a combination of cLA and nitrite. cLA was administered via osmotic mini-pump (20 mg/kg/d) and nitrite was given in drinking water (50 ppm) for a period of 6 weeks. All animal studies were conducted in compliance with the Institutional Animal Use and Care Committee guidelines.

Echocardiography

Transthoracic echocardiography was performed using a VisualSonics (Vevo 770; VisualSonics Inc., Toronto, Ontario, Canada). Left ventricular function was analyzed via the short parasternal axis view. Echocardiograms were performed twice prior to treatment. Measurements were repeated 5-6 weeks after treatment to examine effects of the treatment.

Cell culture and reagents

HUVEC and BAEC cells were obtained from Lonza (Walkersville, MD). HUVEC cells were grown at 37 C in EMB-2 Basal Medium and supplemented with EGM-2 Growth Factors (Lonza). Cells were passed once they reached between 80 and 100% confluence. BAECs were maintained in Medium 199. Experiments were performed on cells at passages 2-10. Once plated, and at appropriate confluence, cells were incubated with serum free medium for 24 hours. Cells were then treated with MeOH or 2.5, 5, or 10 μ M conjugated linoleic acid (cLA).

Western blotting analysis

Heart tissue was snap frozen and stored in -80° C until needed. Tissue was ground with a mortar and pestle, homogenized, and sonicated. Cells were treated for either 6 or 24 hours, then were collected in 1mL of 1X PBS and centrifuged at 1500G for 5 minutes to pellet cells. PBS was removed and the cell pellet was reconstituted in Lysis Buffer. Protein was determined using a Bradford Protein Assay on a Synergy2 by BioTek (Winooski, VT). Using 100 μ g of homogenate, Western Blot analysis was performed using the SDS-PAGE method. 25 μ g of cell lysate was used and Western Blot was performed as previously described. eNOS (1:1000) antibodies were purchased from BD Transduction Laboratories (Franklin Lanes, NJ). All results were normalized to GAPDH (1:3000), purchased from Trevigen (Gaithersburg, MD) or Beta Actin (1:1000), purchased from Sigma Aldrich (St. Louis, MO).

LT PAGE analysis

Heart tissue was snap frozen and stored in -80 C until needed. Tissue was ground with a mortar and pestle, homogenized, and sonicated Protein was determined as previously

described. Reagents (including buffers and gels-5%) were equilibrated in 4 C cold room prior to running. Using 50µg of homogenate, samples containing 50mM triethanolamine, . 2mM BH₄, where indicated, for a final volume of 24µL were incubated at 37 C for 10 minutes. Following incubation, 24µL of 2X sample buffer containing 2-mercaptoethanol was added to the sample. Electrophoresis was performed in a 4 C cold room overnight at 65V. eNOS (1:1000) antibodies were purchased from BD Transduction Laboratories.

NO analysis

Chemiluminescence was used to determine accumulation of nitrite (NO₂⁻) and nitrate (NO₃⁻) via the Sievers 280i Nitric Oxide Analyzer (NOA) (General Electric, Boulder, CO). NO was detected following the reduction of NO₂⁻ with KI and CuSO₄ and NO₃⁻ was detected using vanadium(III) chloride (VCl₃), according to manufacturer's instructions (Sievers). Plasma collected from mice after 6 weeks of treatment, media collected after each treatment and cell lysate were all measured.

Statistical analysis

Multiple comparisons for each variable were performed using analysis of variance (ANOVA) with the post hoc Tukey's or Bonferroni's test. When applicable, the unpaired *t*-test was used to test the significance of each pre-planned comparison. Data was considered statistically significant for *P* < 0.05. All data is presented as mean ± SEM.

Results

Aged mice have decreased cardiac function compared to young mice

To establish baseline myocardial function, echocardiography was performed in 3 month-old mice (untreated). Mice were aged to 10 months and cardiac function was re-assessed to ascertain function in aged mice (Fig. 1A). Analysis of left ventricular function, determined via percent of fractional shortening (%FS), demonstrate that aged WT mice have significantly decreased cardiac function compared to young WT mice (68±3 vs. 35±6, **p*<0.05) (Fig. 1B). Heart rate remained constant throughout echocardiograms across treatment groups (Fig. 1C).

cLA exacerbates cardiac dysfunction in aged mice

At 10 months of age, mice were treated with 20 mg cLA (per kg/d) via osmotic mini pump for 6 weeks. After 6 weeks of treatment, echocardiography was performed to establish cardiac function post treatment (Fig. 2A). Left ventricular function analysis demonstrates that mice treated with cLA have significant dysfunction when compared to age-matched controls, determined by %FS (35±6 vs. 19±9, **p*<0.05) (Fig. 2B). There were no significant weight differences following treatment (data not shown).

cLA decreases intracellular NO levels in Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were treated with increasing concentrations of cLA (2.5, 5, and 10µM) for 6 hours, collected in PBS, and reconstituted in lysis buffer. Given that endothelial cells are a major source of NO, cell lysate was measured for intracellular NO (nitrite and nitrate).

Chemiluminescence analysis demonstrated that cLA reduced levels of both nitrite and nitrate in a dose dependent manner compared to controls (2.7 μ M vs. 1.5 μ M, * p <0.05) (Fig. 3A, B). Moreover, treated media was collected and analyzed for nitrite and nitrate, and showed no change (Fig. 3C). These results indicate that the decrease in NO is due to an intracellular event, and is not media dependent.

cLA decreases intracellular NO levels in Bovine Aortic Endothelial Cells (BAECs)

BAECs were treated with increasing concentrations of cLA (2.5, 5, and 10 μ M) for 6 hours, collected in PBS, and reconstituted in lysis buffer. Based on previous results indicating a loss of intracellular NO in HUVECs, cell lysate was measured for intracellular NO (nitrite and nitrate). Chemiluminescence analysis demonstrated a drop in nitrite only at a concentration of 10 μ M cLA (Fig. 4A), however a similar trend to that of HUVECs was found in nitrate levels (Fig. 4B). NO_x levels in media collected from aforementioned treatments remained unchanged (Fig. 4C).

cLA decreases NOS protein expression in HUVECs, but not in BAECs

Western blot analysis was performed to determine eNOS expression in cell lysate following treatment with increasing concentrations of cLA (Fig. 3D and Fig. 4D). Densitometry results indicated that cLA treatment decreased eNOS protein expression (* p <0.05) in HUVECs, however no differences were observed between concentrations (Fig. 3E). No changes were observed in eNOS protein expression following cLA treatment in BAECs (Fig. 4E).

cLA induces Nox2 protein expression and eNOS monomerization in BAECs

Western Blot Analysis was performed to determine Nox2 expression. Results demonstrated that BAECs had increased Nox2 expression (* p <0.05) following treatment with increasing cLA concentration (Fig. 4D, F). Using LT PAGE, BAECs treated with cLA demonstrated increased eNOS monomer compared to control (Fig. 4G). These results suggest an increase in eNOS uncoupling following cLA treatment. Importantly, BH₄-induced dimerization occurs following incubation.

Sodium nitrite supplementation increases plasma NO_x levels in WT mice

Chemiluminescence analysis performed on plasma collected from mice at the time of sacrifice showed that mice treated with both nitrite and the combination of cLA and nitrite had a considerable increase in nitrite and nitrate levels compared to control and cLA animals (0.13 μ M vs. 0.32 μ M, 0.13 μ M vs. 1.22 μ M, respectively and 42 μ M vs. 184 μ M, 42 μ M vs. 111 μ M, respectively, * p <0.05) (Fig. 5A, B).

cLA decreases plasma NO_x levels in aged WT mice

Chemiluminescence analysis demonstrated that there are significant differences in NO_x levels between 3 month old and 10 month-old untreated mice (22 μ M vs. 44 μ M * p <0.05) (Fig. 5C). Aged mice demonstrate a significant difference in plasma NO_x levels following cLA treatment compared to control treated animals (44 μ M vs 30 μ M # p <0.05) (Fig. 5C). Moreover, 3 month-old mice treated with cLA show no significant differences compared to control treated animals following treatment (Fig. 5C).

cLA decreases eNOS protein expression in aged murine heart, which is rescued following nitrite supplementation

Western blot analysis was performed, and results indicated that animals treated with cLA showed a reduced amount of eNOS compared to controls (Fig. 6A). Following co-treatment with cLA and nitrite, eNOS expression was comparable to that of controls (Fig. 6). Densitometry results show that following normalization with GAPDH, eNOS protein expression is significantly decreased after cLA treatment, however cLA and nitrite co-treatment attenuates the decreased expression (Fig. 6B, where $*p<0.05$ compared to control and $\#p<0.05$, compared to cLA). eNOS protein expression does not change following treatment with cLA in cardiac tissue of young treated mice (data not shown).

cLA induces Nox2 protein expression in aged murine heart, which is rescued with nitrite supplementation

Western blot analysis was performed, and results indicate that animals treated with cLA demonstrate a modest increase from control values. Importantly, mice treated with the combination of cLA and nitrite reveal a decrease in Nox2 expression compared to cLA treated animals ($*p<0.05$, Fig. 6A, C).

eNOS dimerization is decreased in mice treated with cLA

LT PAGE analysis was performed on mouse cardiac tissue. Results indicate that mice treated with cLA have decreased eNOS dimerization compared to control mice, suggesting eNOS uncoupling. This effect is partially rescued with co-administration of cLA and nitrite (Fig. 6D). Notably, uncoupled samples incubated with BH_4 show a marked increase in dimer, indicating BH_4 -induced eNOS dimerization (Fig. 6E).

Nitrite supplementation rescues cLA exacerbated cardiac dysfunction in aged mice

Echocardiography performed on 10 month-old mice co-treated with cLA and nitrite for 6 weeks (20 mg/kg/d cLA and 50 ppm nitrite) demonstrated improved cardiac function compared to mice treated with cLA alone (Fig. 7A). Analysis of left ventricular function, determined via %FS measurements reveal that mice receiving co-administration of cLA and nitrite had improved function compared to cLA-treated mice (19 ± 9 vs. 31 ± 10 , $*p<0.05$) (Fig. 7B).

Discussion

Numerous studies surrounding cLA focus on the potential health benefits of dietary supplementation. However, as this study reveals, there are possible adverse health benefits to cLA supplementation. Previous rodent studies have established that with increasing age there is an increased risk for developing cardiac dysfunction, as indicated by %FS [15]. Using the same parameters, we confirm these findings herein, demonstrating that 10 month-old mice have significantly decreased %FS, resulting in diminished cardiac function compared to 3 month-old mice. In addition, treatment with cLA worsens cardiac dysfunction in aged mice, however no adverse effects were observed in young mice. These results suggest that cardiac dysfunction caused by cLA may only occur with preexisting dysfunction in the myocardium.

Moreover, it is known that decreased NO bioavailability is associated with cardiovascular disease and increasing age [16]. Our results indicate that cLA decreases intracellular NO levels in HUVECs, as well as BAECs. Interestingly, the loss of intracellular nitrite following cLA treatment in BAECs is not as prevalent as that seen in HUVECs. It is possible that this is due to inconsistencies as a result of sample size, as well as the unstable nature of nitrite. Notably, cLA-mediated decreases in intracellular nitrate levels in BAECs were consistent to those seen in HUVECs. Importantly, the overall intracellular nitrate/nitrite levels differ between HUVECs compared to BAECs. Lysate collected from BAECs expressed considerably less NO_x (nitrite and nitrate) than HUVECs. While eNOS protein expression is decreased in HUVECs following cLA treatment, it remains unchanged in BAECs. This difference could be attributed to the measurable differences of NO in the lysate between the two endothelial cell culture models. The level of NO is lower in BAECs, thus the cells are conditioned to survive with less overall NO, and may require significant stress to observe effects similar to those seen in HUVECs. There were no changes observed in mRNA levels or phosphorylation of eNOS (data not shown).

It is important to note that cLA treatment also caused a significant induction of Nox2 protein expression in BAECs. As a result of increased Nox2, increases in ROS could potentially offset levels of NO in this endothelial culture model. Other studies have observed a similar reduction in intracellular NO with a concurring increase in ROS production, following linoleic acid treatment in human aortic endothelial cells (HAECs) [17]. As determined by LT PAGE, our results indicate that treatment with cLA in endothelial cells increases monomerization of eNOS compared to controls. These results indicate that 5μM cLA treatment uncouples eNOS in endothelial cells, which may cause the production of superoxide, as opposed to NO. Similar results have been reported, where Nox-mediated peroxynitrite-dependent uncoupling of eNOS can occur [18].

Importantly, our results show that plasma levels of nitrite can be increased from control values when mice are treated with the combination of cLA and nitrite (or nitrite alone). Moreover, our data demonstrate that untreated 10 month-old aged mice have significantly more plasma NO than 3 month-old mice. However, following treatment with cLA, plasma NO levels are significantly decreased compared to age-matched controls. Interestingly, 3 month-old mice show no significant difference between control and cLA treated animals. Previous studies in humans suggest that there is an increase in serum NO_x levels as a patient's age increases from their 20s to their 30s [19]. The authors speculate that this could be due to compensation for the loss of endothelial-derived NO (which is reduced with age), by pulmonary artery derived NO (which is not reduced with age) [19]. Notably, it has been demonstrated previously that mice 6 months of age are equivalent to a 30 year old human and 13 month-old mice are equivalent to a 45 year-old human [20]. Based on these findings, it could be assumed that the 10 month-old mice used in this study would have an approximate human age in the late 30s, thus our findings of increased plasma NO_x in 10 month-old mice compared to 3 month-old animals are consistent with previous literature reports.

Additionally, cLA diminished eNOS protein expression in murine tissue can be restored to control levels following nitrite supplementation. However, Nox2 protein, which is modestly

induced by cLA, is in turn, significantly reduced with co-treatment of cLA and nitrite, compared to cLA values. Taken together, these results indicate that cLA supplementation lowers physiological NO in the vasculature via an increase in Nox2, while combination treatment of cLA and nitrite mediates an increase in cardiac NO production via a decrease in Nox2 and a subsequent increase in eNOS. Previous studies have demonstrated that a loss of NO bioavailability results in an increase in oxidative stress due to decreased NO-dependent free radical scavenging and that sodium nitrite supplementation is able to decrease NADPH oxidase activity, which may subsequently decrease oxidative stress [21].

Moreover, LT PAGE analysis illustrates that mice treated with cLA have a decrease in eNOS dimerization compared to control treated mice. Importantly, the monomer can be shifted to dimer following either nitrate supplementation in vivo or BH₄ incubation in vitro. As previously mentioned, a decrease in eNOS dimerization indicates uncoupling, which results in diminished NO production. Co-administration of cLA and nitrite also restored cLA-induced cardiac dysfunction to control levels, demonstrating that decreased or dysfunctional eNOS could contribute to decreased cardiac function.

Recent studies have shown that eNOS-expressing endothelial progenitor cells (EPCs) transplantation in WT mice that suffered myocardial infarction (MI) provided protection and lessened infarct size while also preserving %FS, possibly through increasing angiogenesis [22]. Results also demonstrate that mice suffering MI had significantly decreased eNOS protein expression and cardiac tissue NO levels. Furthermore, eNOS-EPCs transplant group showed increases in both eNOS protein and cardiac tissue NO levels [22]. Previously, we reported that cLA exacerbates cardiac dysfunction following MI [23]. However, co-treatment with cLA and nitrite preserves cardiac function following MI, through increased miR-499 and inhibition of p53-Drp1 mediated apoptotic signaling [23]. Results reported here further reinforce the importance of functional eNOS and physiological NO in preservation of cardiac function, especially in cLA-treated mice.

It is essential to point out that weight was monitored during the current study, due to the numerous reports of weight loss benefits of cLA. As previously mentioned, both prominent cLA isomers have been shown to have positive weight management effects. The c9, t11 isomer is specifically implicated in limiting weight gain and preserving muscle mass. As this study focused solely on the c9, t11 isomer, our results are consistent with previously published findings, where there are no weight differences between treatment groups.

Overall our study reinforces the concept that maintaining physiological levels of NO is important for preserving cardiac function. We demonstrate that aged mice have considerable cardiac dysfunction when compared to young mice. Administration of cLA further exacerbates the aforementioned cardiac dysfunction, cautioning that various reports of beneficial health effects should be examined more closely. Moreover, it has been proven in numerous studies that eNOS is an essential enzyme in regard to preservation of cardiac function. When treated with cLA, aged mice have decreased eNOS protein expression coupled with increased eNOS monomerization, indicating that cLA could alter the function and expression of eNOS. Taken together, these findings suggest that cLA treatment could lead to a subsequent decrease in overall NO production in the vasculature. Contrary to our

original hypothesis, it is clear that while it may be helpful in many other disease states, there are possible detrimental side effects of cLA supplementation in aged individuals.

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Highlights

- Aged mice have significant cardiac dysfunction compared to young mice
- cLA exacerbates cardiac dysfunction in aged mice
- cLA diminishes eNOS protein in HUVECs and murine cardiac tissue
- eNOS protein expression is rescued with nitrite supplementation
- Cardiac dysfunction is attenuated by nitrite supplementation in aged mice

*p<0.05

Fig. 1.

Aged mice have significant cardiac dysfunction compared to young mice. Echocardiograms in M mode show cardiac dysfunction in aged mice (A). 10 month-old mice have a significantly lower %FS than 3 month old mice (68 ± 3 vs. 35 ± 6 , $*p<0.05$) (B).

Fig. 2. cLA significantly decreases cardiac function in aged mice. Echocardiograms in M mode demonstrate significant dysfunction in cLA treated mice compared to controls (A). 10 month-old mice treated with cLA have significantly lower %FS compared to controls (35±6 vs. 19±9, *p<0.05) (B). Heart rate remained constant during heart function analysis (C).

Fig. 3. cLA decreases intracellular nitric oxide (NO) levels in human umbilical vein endothelial cells (HUVECs). Nitrite (A) and nitrate (B) levels are significantly decreased in HUVECs treated with increasing concentrations of cLA, (*p<0.05), while NO levels in media remain unchanged (C). Consequently, eNOS protein expression was decreased following treatment with cLA (D) and significantly reduced at 2.5 μM (*p<0.05) (E).

Fig. 4. cLA decreases intracellular nitric oxide (NO) levels in bovine aortic endothelial cells (BAECs). Nitrite (A) levels are reduced with a 10μM cLA treatment (*p<0.05), and nitrate (B) levels are reduced in samples treated with both 5μM and 10μM cLA in BAECs, while NO levels in media remain unchanged (C). eNOS protein expression is unaffected following cLA treatment in BAECs (D and E). Treatment with increasing concentrations of cLA induces Nox2 protein expression in BAECs (D). Nox2 expression was significantly increased from controls, although no differences were seen between concentrations of cLA (*p<0.05) (F). Subsequently, LT PAGE analysis demonstrated that cLA treatment increased eNOS monomerization in BAECs compared to control treated samples (G), indicating uncoupling of eNOS.

Fig. 5.

Co-administration of cLA + nitrite increases plasma nitrite in WT mice. Mice treated with both nitrite and the combination of cLA and nitrite, demonstrate a significant increase in plasma nitrite levels compared to control or cLA treated animals (* $p < 0.05$) (A). Nitrite and combination cLA and nitrite treated mice also demonstrated a significant increase in plasma nitrate levels (* $p < 0.05$) (B). 10 month-old mice have significantly more plasma NO compared to 3 month old mice (* $p < 0.05$) (C). Following cLA treatment, 10 month-old mice have significantly reduced NO levels from age matched controls (# $p < 0.05$) (C).

Fig. 6.

cLA decreases eNOS protein expression in murine cardiac tissue. 10 month-old mice treated with cLA demonstrate a decrease in eNOS protein compared to controls (A). Quantitation of eNOS expression, normalized to GAPDH, indicates that cLA treatment causes a significant decrease in eNOS protein ($*p<0.05$, compared to control) and co-administration of cLA and nitrite resulted in a significant increase of eNOS expression ($\#p<0.05$, compared to cLA) (B). cLA induces Nox2 expression in murine cardiac tissue. 10 month-old mice treated with cLA demonstrate a modest increase in Nox2 expression (A). Moreover, co-administration of cLA and nitrite results in a significant decrease when compared to cLA-treated mice ($*p<0.05$) (A, C). Animals treated with cLA indicate a loss of eNOS coupling. Mice treated with cLA show a marked decrease in eNOS dimerization, compared to controls. This is moderately corrected with the combination of cLA and nitrite. Importantly, incubation with BH₄ shifts all samples towards the dimer form, indicating coupling of eNOS.

*p<0.05

Fig. 7. Nitrite supplementation rescues cLA-exacerbated cardiac dysfunction in aged mice. Echocardiograms in M mode demonstrate that cLA-mediated cardiac dysfunction is attenuated with co-administration of cLA and nitrite (A). %FS is rescued in cLA + nitrite treated animals (B).