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Original Contribution

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 U.S. FDA. Numerous studies suggest that cLA mediates protection against diseases including cancer, diabetes, atherosclerosis, immune function, and obesity. Based upon these reports, it was hypothesized that supplementation with 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 percentage of fractional shortening. 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 cotreated with cLA and nitrite. Taken together, these results suggest that cLA-mediated cardiac injury can be circumvented by nitrite supplementation in a murine model of aging.

Conjugated linoleic acid (cLA) refers to a group of naturally occurring isomers of linoleic acid characterized by conjugated double bonds, which may occur at several different positions [1]. There are two prominent isomers, of which the *cis*-9, *trans*-11 isomer is naturally present in ruminant meat and dairy products [2], whereas *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 U.S. Food and Drug Administration (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 suggest 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, *t*10, *c*12 is known to reduce adiposity, as *c*9, *t*10 simply lessens body weight gain. However, recent studies have shown that *t*10, *c*12 can increase the expression of leptin, whereas *c*9, *t*11 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 *c*9, *t*11 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, and during an acute-phase or inflammatory immune response, cLA can alter immune function through decreased production of eicosanoids as well as suppression of proinflammatory cytokines [7]. However, other reports suggest that cLA has no effect on immune function in healthy individuals [7].

Although 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 with 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.

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Inflammatory vascular diseases are often marked with a loss of nitric oxide (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].

Although eNOS is important for NO bioavailability in the vasculature, it is not the only enzyme that 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 supplementation with inorganic nitrite can counteract adverse effects caused by a lack of 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 few or no side effects at nontoxic 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 aged 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 owing to both structural and functional changes. Moreover, these changes can affect the modulation of cardiovascular diseases [14]. Considering 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 *c*9, *t*11 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/6 J mice were obtained from The Jackson Laboratory at 8–10 weeks of age and aged to 38 weeks before 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, cLA, nitrite, and a combination of cLA and nitrite. cLA was administered via osmotic mini-pump (20 mg/kg/day) 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 ultrasound system (Vevo 770; VisualSonics, Inc., Toronto, ON, Canada). Left-ventricular function was analyzed via the short parasternal axis view. Echocardiograms were performed twice before treatment. Measurements were repeated 5 or 6 weeks after treatment to examine the effects of the treatment.

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) were obtained from Lonza (Walkersville, MD, USA). HUVECs 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 the appropriate confluence, cells were incubated with serum-free medium for 24 h. The cells were then treated with MeOH or 2.5, 5, or 10 μM cLA.

Western blotting analysis

Heart tissue was snap-frozen and stored at −80 °C until needed. Tissue was ground with a mortar and pestle, homogenized, and sonicated. Cells were treated for either 6 or 24 h and then were collected in 1 ml of 1 × phosphate-buffered saline (PBS) and centrifuged at 1500*g* for 5 min to pellet the 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, USA). Using 100 μg of homogenate, Western blot analysis was performed using the SDS–PAGE method. Cell lysate (25 μg) was used and Western blot was performed as previously described. eNOS (1:1000) antibodies were purchased from BD Transduction Laboratories (Franklin Lanes, NJ, USA). All results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:3000), purchased from Trevigen (Gaithersburg, MD, USA) or β-actin (1:1000), purchased from Sigma–Aldrich (St. Louis, MO, USA).

Low temperature (LT) PAGE analysis

Heart tissue was snap-frozen and stored at −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 a 4 °C cold room before running. Using 50 μg of homogenate, samples containing 50 mM triethanolamine, 0.2 mM tetrahydrobiopterin (BH_4), where indicated, for a final volume of 24 μl, were incubated at 37 °C for 10 min. After incubation, 24 μl of 2 × sample buffer containing 2-mercaptoethanol was added to the sample. Electrophoresis was performed in a 4 °C cold room overnight at 65 V. eNOS (1:1000) antibodies were purchased from BD Transduction Laboratories.