Sustained Release Nitrite Therapy Results in Myocardial Protection in a Porcine Model of Metabolic Syndrome with Peripheral Vascular Disease

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Short Title: Nitrite Improves Coronary Vascular Reactivity in Critical Limb Ischemia

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Abstract

**Background:** Metabolic syndrome (MetS) reduces endothelial nitric oxide (NO) bioavailability and exacerbates vascular dysfunction in patients with pre-existing vascular diseases. Nitrite, a storage form of NO, can mediate vascular function during pathological conditions when endogenous NO is reduced. The aims of the present study were to characterize the effects of severe MetS and obesity on dyslipidemia, myocardial oxidative stress, and endothelial nitric oxide synthase (eNOS) regulation in the obese Ossabaw swine (OS) model; and to examine the effects of a novel, sustained release sodium nitrite formulation (SR-Nitrite) on coronary vascular reactivity and myocardial redox status in the obese OS subjected to critical limb ischemia (CLI).

**Methods/Results:** Following 6 months of an atherogenic diet, Obese OS displayed a MetS phenotype. Obese OS had decreased eNOS functionality and NO bioavailability. In addition, obese OS exhibited increased oxidative stress and significant reduction in antioxidant enzymes. The efficacy of SR-Nitrite therapy was examined in the obese OS subjected to CLI. Following 3 weeks of treatment, SR-Nitrite (80 mg/kg/d b.i.d. p.o.) increased myocardial nitrite levels and eNOS function. Treatment with SR-Nitrite reduced myocardial oxidative stress while increasing myocardial antioxidant capacity. **Ex vivo** assessment of vascular reactivity of the left anterior descending coronary artery segments demonstrated marked improvement in vasoreactivity to sodium nitroprusside but not to substance P and bradykinin in SR-Nitrite treated animals compared to placebo. **Conclusion:** In a clinically relevant, large animal model of MetS and CLI, treatment with SR-Nitrite enhanced myocardial NO bioavailability, attenuated oxidative stress, and improved **ex vivo** coronary artery vasorelaxation.
Introduction

Metabolic syndrome (MetS) is a multifactorial disease that is characterized by a number of risk factors including obesity, hypertension, dyslipidemia, and hyperglycemia (27). Clinically, MetS is associated with an increase risk for the development of cardiovascular disease. In patients with pre-existing atherosclerotic vascular disease, such as peripheral artery disease (PAD), MetS exacerbates vascular damage by impairing vascular endothelial and smooth muscle function (37). Furthermore, PAD patients with MetS have an increased incidence of acute coronary syndromes resulting in significant cardiovascular morbidity and mortality (5, 16, 20, 21).

One of the earliest manifestations of cardiovascular diseases including PAD is a lost of endothelial function characterized by dysfunction of endothelial nitric oxide synthase (eNOS) and reduced nitric oxide (NO) bioavailability (7, 19, 44). Our laboratory has recently published data (40) demonstrating significant reductions in circulating NO bioavailability in patients with critical limb ischemia (CLI), a severe manifestation of PAD.

Nitrite is recognized as an important physiologic storage reservoir of NO in the blood and tissues that protects various organs against ischemic injury (10, 12, 31). During pathological conditions, nitrite is rapidly reduced to NO to restore NO levels and promote organ homeostasis and survival (10, 12, 31). Moreover, endogenous nitrite has been shown to exert endocrine actions during ischemic injury via transport in the circulation and subsequent metabolism in distant organs to mediate cytoprotection (12). In rodent models of cardiovascular disease and aging, daily nitrite supplementation increases myocardial NO levels (2, 6), reverses vascular endothelial dysfunction (43),
and attenuates oxidative stress (8). These data suggest a promising therapeutic potential of sodium nitrite in cardiovascular disease states. One of the major limitations with the use of sodium nitrite for the treatment of chronic cardiovascular diseases is the relatively short half-life of sodium nitrite and the inability to maintain pharmacologically relevant levels of nitrite and NO for sustained periods. (30). In order to overcome this severe limitation, we recently investigated the effects of a novel, sustained release formulation of sodium nitrite (SR-Nitrite) in a clinically relevant model of CLI (40). In this study obese Ossabaw swine were subjected to CLI for 35 days and treated with SR-Nitrite starting at day 14. In this study we failed to observed significant improvements in hindlimb perfusion, revascularization, or perfusion pressures. However, we did observe significant increases in NO bioavailability in the plasma and the ischemic skeletal muscle at 35 days post CLI injury coupled with reductions in oxidative stress and induction in pro-angiogenic signaling (40).

At present, there are no studies evaluating the effects of obesity/Mets in the presence of peripheral vascular disease on myocardial oxidative stress and coronary artery vasomotion. The goals of the current study were two-fold. Initially, we sought to assess the extent of eNOS dysregulation, NO insufficiency and myocardial oxidative stress in a clinically relevant model of obesity and MetS (i.e. obese Ossabaw swine model). Subsequently, we evaluated the effects of the SR-Nitrite formulation on blood pressure, myocardial oxidative stress, NO bioavailability, and coronary artery vasoreactivity in the obese Ossabaw swine subjected to CLI.
Methods

Animals. Female Ossabaw swine (OS), 12-14 months of age, were obtained from Indiana University and handled in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health and according to the guidelines of the Emory University Institutional Animal Care and Use Committee.

Ossabaw Swine Lean versus Obese Study. Female OS (6-8 months of age) were fed either 725 g/day lean diet (5L80, Lab Diet) or 1 kg/day atherogenic obese diet (KT324, Test Diet) comprised of 2% cholesterol, 17% coconut oil, 2.5% corn oil, and 0.7% sodium cholate for 6 months. Following 6 months of feeding, heparin (200 U/kg IV) was injected and allowed to circulate for approximately 5 minutes then animals were euthanized while under deep inhalant anesthesia (isoflurane, 5%). Heart and blood were collected.

Serum Cholesterol and Biomarkers. Lipid profiles and asymmetric dimethylarginine (ADMA) quantification was performed by Atherotech Diagnostics Lab (Birmingham, AL).

Critical Limb Ischemia (CLI) Model. Obese OS subjected to CLI investigated in this study were a part of another experimental study that investigated the efficacy of SR-Nitrite in skeletal muscle (40). Animals were sedated with medications that included ketamine (15 mg/kg) in combination with xylazine (1 mg/kg) for sedation and to aid induction, animals were endotracheally intubated and maintained on isoflurane in oxygen. Pre-operative analgesic buprenorphine (0.15 mg for each pig) was given along
with peri-operative antibiotics cefazolin (1000 mg). Post-op analgesics carprofen (10mg) and buprenorphine (0.15 – 0.3 mg) were given immediately after the procedure. Post-operative day 1 pigs received additional analgesics buprenorphine (0.3 mg) in combination with carprofen (100 mg). All pigs were monitored during the procedure for heart rate, respiration, spO2, ETCO2, fluid volume and body temperature. Activated clotting time was measured with a Hemachron after the arterial sheath was placed and heparin given.

Using sterile conditions and under fluoroscopic guidance, an 8-French introducer (Maximum Hemostasis Introducer 23 cm ACT Sheath, St. Jude Medical, St. Paul, MN) was inserted into the right external carotid artery, advanced into the abdominal aorta and placed just above the aortoiliac bifurcation. Heparin (300 U/kg) was administered and activated clotting time was monitored throughout the procedure to ensure clotting times of at least 250 seconds. A percutaneous guidewire (0.035 x 230 cm, Rosen Starter; Boston Scientific, Natick, MA) was positioned in the right external iliac artery. A 7-French delivery guide catheter (MACH 1 MP1; Boston Scientific, Natick, MA) containing a self-expanding endoluminal endoprosthesis consisting of an expanded polytetrafluoroethylene (ePTFE) lining with an external nitinol stent (6 mm x 15 cm; Viabahn; W.L. Gore and Associates, Inc., Flagstaff, AZ) was advanced, positioned within the right external iliac artery, and deployed. Contrast arteriography was performed to evaluate vessel patency. Following placement of the ePTFE-lined endoprosthesis, a self-expanding nitinol mesh occlusion device (8 x 7 mm; Amplatzer Vascular Plug II; St. Jude Medical, St. Paul, MN) was loaded onto the delivery guide catheter, advanced, positioned within the right external iliac artery and deployed within
the proximal portion of the ePTFE-lined stent. An arteriogram was performed to verify occlusion. The guide wire, guide catheter, and introducer sheath were removed and the left external carotid artery was ligated. The incision was closed and the animal was allowed to recover and buprenorphine (0.05 mg/kg) was administered for analgesia. The ePTFE-lined endoprosthesis served to inhibit recanalization of the artery by collateral vessels, typically observed in animal models of critical limb ischemia.

Sodium Nitrite Therapy. Sustained release (SR) sodium nitrite tablets and placebo tablets were obtained from TheraVasc Inc. (Cleveland, OH). Animals received placebo (n=6) or 80 mg of SR-Nitrite (n=7) by mouth (p.o.) twice daily (b.i.d.). Treatment was initiated at 14 days following the induction of CLI and continued for 21 days.

DSI Radiotelemetry Implantation. Prior to induction of CLI, obese OS (n=8) underwent surgical implantation of an arterial BP radiotelemeter (TA11PA-D70; Data Sciences International, St Paul, MN) to assess the extent of hypertension in the obese Ossabaw swine. Anesthesia was induced and maintained as described above. The BP telemeter body was implanted subcutaneously dorsally in the neck, with the telemeter catheter inserted 10 cm into the left carotid artery. Telemetry equipment (Data Sciences International) was set up to monitor hemodynamics. The BP telemeter signal was received by a receiver (RLA1020) positioned in a protective case on the floor of the holding pen. The strongest signal was selected by a multiplexer (RMX10) and passed to an analog adapter (R11CPA), which provided a calibrated voltage output after correcting for atmospheric pressure using an ambient pressure monitor (APR-1). The calibrated signal was then recorded using a
computerized data acquisition system. The BP signal was sampled for 10 s at 30 s intervals, mean arterial blood pressure (MABP) during this sample period were computed and stored for offline analysis.

Euthanasia and Tissue Collection. Following 35 days of CLI, the Obese OS were euthanized and blood, myocardial tissue, and coronary artery segments were obtained. Heparin (200 U/kg IV) was injected and allowed to circulate for approximately 5 minutes then animals were euthanized while under deep inhalant anesthesia (isoflurane, 5%). Heart and coronary arteries were collected.

Measurement of NO metabolites. Nitrite concentrations were quantified in ventricular tissue biopsies by ion chromatography (ENO20 Analyzer; Eicom, Kyoto, Japan).

cGMP RIA. Coronary artery cGMP concentrations were quantified as previously described (2).

Western Blot Analysis. Ventricular tissue was used for Western blot analysis, performed as described previously (23). The following primary antibodies were used: VEGF, CD31 (Novus Biologicals; Littleton, CO), eNOS$^{\text{Ser}177}$ (Abcam; Cambridge, MA), eNOS, eNOS$^{\text{Thr}495}$ (Cell Signaling; Danvers, MA), and GAPDH (Santa Cruz Biotechnology; CA).
Real-time PCR. Ventricular mRNA was quantified using TaqMan primers from Life Technologies and performed as previously described (36).

Determination of Protein Carbonyl Content. Protein carbonyl content from ventricular tissue was measured as described previously (23).

Measurement of Malondialdehyde (MDA) Levels. MDA levels were measured in ventricular tissue as previously described (23).

Functional Assessment of Isolated Coronary Rings. Isolated coronary artery studies were performed in obese OS and in lean, non-ischemic Yorkshire control pigs. The CLI experiments involved obese OS receiving either placebo or SR-Nitrite (80 mg p.o. b.i.d.). The obese OS vascular reactivity responses were compared to the non-ischemic lean Yorkshire responses. Coronary arteries from Yorkshire pigs (3 months; Palmetto farms) were used as a reference for a non-ischemic control. Left anterior descending (LAD) arteries were dissected and cleaned of surrounding perivascular adipose tissue and collected into ice-cold Krebs buffer. Arteries were cut into 3 mm rings and mounted in organ baths with warm Krebs buffer for isometric tension studies. Coronary arteries were placed under 0.5 g of tension for 90-120 minutes to obtain a stable and optimal baseline passive tension. Arteries were stimulated initially with 40 mM and then 100 mM potassium chloride (KCl); n = 2 Non-ischemic control; n = 6 CLI + Placebo; n = 6 CLI + SR-Nitrite) to assess vessel viability and contractile responsiveness. After a series of washes, rings were pre-contracted with prostaglandin F2α (PGF2α; 30 µM). At peak constriction, vascular function was assessed by the addition
of graded concentrations of sodium nitroprusside ($10^{-9}$ to $10^{-5}$ μM; n = 2 Non-ischemic control; n = 6 CLI + Placebo; n = 5 CLI + SR-Nitrite), bradykinin ($10^{-9}$ to $10^{-5}$ μM; n = 2 Non-ischemic control; n = 6 CLI + Placebo; n = 5 CLI + SR-Nitrite), or substance P ($10^{-11}$ to $10^{-8}$ μM; n = 2 Non-ischemic control; n = 6 CLI + Placebo; n = 4 CLI + SR-Nitrite), to the tissue bath.

**Statistical Analysis.** All data in this study are expressed as the mean ± SEM. Differences in data between the groups were compared using Prism 6 (GraphPad Software). A Student’s unpaired, two-tailed t-test when comparison was between two groups (i.e. lean OS vs. obese OS or CLI + Placebo vs. CLI + SR-Nitrite). For ambulatory MABP, 2-way ANOVA was used to test the effects of placebo and SR-Nitrite therapy over time. For isometric tension dose-dependent studies, 2-way ANOVA was used to test the effect of treatment and various vasodilators on coronary relaxation response. For maximal relaxation, a 1-way ANOVA was used to test the effect of the treatment and the maximum dose of vasodilator on the maximum vasorelaxation. When statistical significance was found with ANOVA, a tukey multiple comparison test was performed. A p value of < 0.05 was considered statistically significant.

**Results**

The obese OS is a well-characterized model of central obesity and MetS (11, 35). The data reported in this paper represent data collected from 2 independent experimental studies. Initial experimental experiments evaluated the cardiovascular and MetS phenotype in the obese OS in comparison to the lean OS. The results from the first
study are summarized in *Figures 1-5*. The second study reported in this paper is a subset of study in which we investigated the efficacy of a novel extended release formulation of nitrite (i.e. SR-Nitrite) in a CLI model (40). The current study, which is presented in this paper, is focused on the effects of SR-Nitrite on myocardial oxidative stress, coronary vascular reactivity, and systemic hemodynamics in the obese OS subjected to CLI. These results are summarized in *Figures 6-11*.

**Characterization of the MetS in the obese Ossabaw.** Following 6 months of a high fat, high cholesterol diet, obese OS exhibited characteristics of a MetS phenotype (*Figures 1 and 2*) when compared to lean controls. Obese OS displayed a significant (*p* < 0.01) increase in body weight (*Figure 1A*) compared to lean OS (73.0 ± 5.0 vs. 40.0 ± 2.0 kg). There was no significant difference in plasma insulin levels (*Figure 1C*); however blood glucose (*Figure 1B*) was elevated (*p* < 0.05) in the Obese OS (80.2 ± 4.0 mg/dL) compared to the lean OS (67.4 ± 4.6 mg/dL). Obese OS animals exhibited 2-fold higher circulating levels (53.5 ± 9.1 U/L) of alanine aminotransferase (ALT; *Figure 1D*; *p* < 0.05) compared to lean OS (24.3 ± 1.2 U/L). Lee *et al.* (24) reported that following 6 months of feeding with a very similar high fat, high cholesterol diet the OS displayed abnormal fatty liver histology that is similar to human nonalcoholic steatohepatitis. Therefore, we suspect that increases in ALT that we observed in the present study are related to steatohepatitis.

The obese diet resulted in significant dyslipidemia in the obese OS as shown in *Figure 2*. Circulating cholesterol levels were significantly (*p* < 0.0001) elevated in the Obese OS (508.5 ± 78.9 mg/dL) compared to lean OS (66.4 ± 2.7 mg/dL). Similarly, obese OS
exhibited significant (p < 0.05) elevations in plasma triglycerides (Figure 2B) versus the lean OS (59.1 ± 13.4 mg/dL vs. 25.4 ± 2.7 mg/dL). Although there was no significant difference between the lean OS and obese OS in terms of HDL cholesterol (Figure 2D), circulating LDL cholesterol (Figure 2C) was increased (p < 0.01) in the obese OS (290.0 ± 51.0 mg/dL) compared to the lean OS (26.6 ± 2.1 mg/dL). VLDL cholesterol levels (Figure 2E) demonstrated significantly (p < 0.01) higher VLDL in the obese OS (26.0 ± 4.0 mg/dL) versus lean OS (10.5 ± 0.2 mg/dL).

Ossabaw Swine, myocardial eNOS dysregulation, and NO deficiency. Experiments were performed to investigate the extent of myocardial eNOS coupling in both lean and obese OS using Western blot techniques. It is well appreciated that phosphorylation of serine\textsuperscript{1177} on eNOS activates the enzyme to increase NO production, while phosphorylation of threonine\textsuperscript{495} inactivates the enzyme and attenuates NO production (4, 26, 34). Western blot analysis of ventricular tissue biopsies obtained from lean and obese OS were similar in total eNOS protein expression (Figure 3B). Obesity and MetS resulted in a significant reduction in phosphorylation of eNOS at the primary activation site, serine\textsuperscript{1177} (Figure 3C) in the obese OS compared to lean OS hearts (0.5 ± 0.1 vs. 1.0 ± 0.1 RI, obese OS vs. lean OS, respectively; p < 0.01). Moreover, obese OS exhibited a marked increase in phosphorylation of the eNOS inhibitory site, threonine\textsuperscript{495} (Figure 3D) (2.6 ± 0.2 vs. 1.1 ± 0.3 RI, obese OS vs. lean OS, respectively; p < 0.05). Myocardial tissue nitrite levels were measured as a surrogate index of NO bioavailability in the heart (Figure 3E). Obese OS displayed reduced cardiac nitrite levels when compared to lean OS (0.8 ± 0.08 vs. 2.2 ± 0.3 μM, obese OS vs. lean OS, respectively;
p < 0.01). In addition, NO-mediated activation of guanylyl cyclase and downstream signaling as measured by cyclic guanosine monophosphate (cGMP) (Figure 3F) was significantly (p < 0.05) decreased in the coronary arteries of the obese OS (2.0 ± 2.0 pmol/g) compared to lean OS animals (8.9 ± 2.5 pmol/g).

Obese OS exhibit altered redox status in the heart. The reductions in eNOS functionality along with a concomitant reduction in nitrite in the obese OS may be mediated by several mechanisms such as excessive oxidative stress and/or increased levels of endogenous eNOS inhibitors. In order to determine the overall redox status in obese OS hearts, we measured the extent of oxidative stress in myocardial tissue samples using malondialdehyde (MDA) and carbonyl as an overall indices of oxidative stress. These data are presented in Figure 4. Cardiac MDA levels were similar between the obese OS and lean OS swine (Figure 4A). Obese OS (3.1 ± 0.5 nmol/protein) did exhibit significantly (p < 0.05) elevated myocardial carbonyl protein levels (Figure 4B) compared to lean OS (2.1 ± 0.2 nmol/protein). Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of eNOS function via its interaction with the L-arginine binding site and thereby inhibits NO production (9). Interestingly, plasma ADMA (Figure 4C) levels were significantly (p < 0.05) higher in obese OS (2.3 ± 0.1 μM/L) compared to lean OS (1.6 ± 0.2 μM/L).

We also measured the levels of mRNA for critical antioxidant enzymes in myocardial tissue from lean and obese OS (Figure 5) to further evaluate the extent of oxidative stress. Glutathione peroxidase (GPx) 1 (Figure 5A) and catalase (Figure 5B) mRNA levels were not statically different between the study groups; however, obese OS had a
significant reduction in hemeoxygenase (HO) – 1 (*Figure 5D*) mRNA in the heart when compared to lean OS (2.1 ± 0.3 vs. 5.8 ± 0.9 AU; p < 0.01). In contrast, qPCR analysis of superoxide dismutase (SOD) 2 mRNA (*Figure 5C*; p < 0.05) revealed a marked increase in obese OS hearts (1.2 ± 0.1 AU) compared to lean OS (0.7 ± 0.1 AU).

Investigation of hypertension and coronary vascular reactivity in obese OS following nitrite therapy. By utilizing a severe model of peripheral vascular disease, we sought to investigate the effects MetS in the setting of CLI on myocardial redox status and coronary vascular function and also to evaluate the potential cytoprotective effects of nitrite in this severe model of cardiovascular disease. The results of these experiments are summarized in *Figures 6-11*.

Hypertension in the obese Ossabaw Swine and the effects of SR-Nitrite. Utilizing the obese OS swine, CLI injury was surgically induced in the right external iliac and sustained for 35 days as depicted in the experimental protocol outlined in *Figure 6*. SR-Nitrite (80 mg p.o. b.i.d.) or placebo therapy was initiated on day 14 following CLI surgery. To ensure that SR-Nitrite therapy did not induce systemic hypotension, mean arterial blood pressure (MABP) was assessed by radiotelemetry. Ambulatory AM and PM averages of MABP are shown in *Figure 6B* revealed that obese OS exhibits a significant hypertensive phenotype. A major concern with the chronic use of nitrite and NO-based therapeutics is the potential for significant and potentially dangerous reductions in systemic blood pressure related to increased circulating NO levels and profound vasodilation. SR-Nitrite therapy at the dosage investigated in this study failed
SR-Nitrite therapy enhances myocardial eNOS function and NO bioavailability in the Obese OS swine following CLI. Obesity and MetS in the obese OS results in dysregulation of eNOS with increased phosphorylation of the enzyme’s inhibitory site and reduction at the active site. This profound inhibition of eNOS results in reductions in NO bioavailability and acceleration in vascular and myocardial pathology. We next evaluated whether SR-Nitrite therapy restored physiological regulation of myocardial eNOS via alterations in phosphorylation and potentially increased myocardial NO bioavailability in the obese OS following the surgical induction of CLI (Figure 7).

Western blot analysis of heart tissue revealed that SR-Nitrite therapy had no significant effect on total eNOS compared to placebo (Figure 7B). Fourteen days of SR-Nitrite resulted in a significant increase in phosphorylation at eNOS Ser1177 (Figure 7C) compared to the Placebo (1.5 ± 0.1 vs. 0.9 ± 0.2 RI, CLI + SR-Nitrite vs. CLI + Placebo, respectively; p < 0.05). Furthermore, SR-Nitrite increased myocardial nitrite levels (Figure 7E; p < 0.05) in the CLI + SR-Nitrite group (1.9 ± 0.2 nmol/mg of protein) compared to the CLI + Placebo (1.2 ± 0.3 nmol/mg of protein). Interestingly, there was no change in eNOS Thr495 phosphorylation between the CLI + SR-Nitrite and CLI + Placebo groups (Figure 7D).

SR-Nitrite therapy reduced oxidative stress in the obese OS following CLI. We next evaluated the effects of chronic SR-Nitrite treatment on myocardial oxidative stress
in the obese OS following CLI injury (Figure 8). Cardiac levels of MDA (Figure 8A) were significantly (p < 0.05) reduced in the CLI + SR-Nitrite (3.3 ± 0.2 nmol/mg of protein) compared to CLI + Placebo (4.1 ± 0.3 nmol/mg of protein). Similarly, carbonyl protein levels (Figure 8B) in CLI + SR-Nitrite hearts (20.5 ± 0.8 nmol/mg of protein) were significantly (p < 0.05) attenuated compared to the placebo (25.9 ± 1.5 nmol/mg of protein). SR-nitrite therapy (23.7 ± 2.9 AU) increased myocardial GPx-1 (Figure 8C; p < 0.05) levels in the CLI + SR-Nitrite compared to placebo controls (14.2 ± 1.7 AU). Although there was a trend toward increase, there was no difference in myocardial catalase, SOD 1, or SOD 2 (Figure 8D-F) between the CLI + SR-Nitrite and CLI + Placebo groups (p = NS for all between study groups).

SR-Nitrite therapy improves coronary vascular reactivity in the setting of obese MetS and CLI. One of the primary goals of this study was to examine the effects of chronic administration of SR-Nitrite on coronary vascular reactivity in vitro. The left anterior descending (LAD) arteries were removed from lean non-ischemic control (Yorkshire domestic farm pigs) and from the CLI obese OS receiving placebo or SR-Nitrite for 21 days. These data are presented in Figures 9-11. The contraction response (Figure 9) to potassium chloride (KCl) and prostaglandin F2α (PGF2α) demonstrates differences between the non-ischemic control and the CLI obese OS. Using the lean non-ischemic Yorkshires as a reference, we observed a 49% reduction in contraction of the CLI obese OS coronaries at concentrations of 40 mM KCl (Figure 9A) and 100 mM KCl (Figure 9B) compared to the non-ischemic controls. The LAD contraction response to PGF2α (Figure 9C) was attenuated by 72% in the CLI obese
OS compared to the non-ischemic controls (p < 0.001). There was no significant
difference between the CLI + Placebo compared to the CLI + SR-Nitrite (p = NS
between groups).

Administration of sodium nitroprusside (SNP) resulted in a dose-dependent relaxation
from PGF2α - induced contraction in non-ischemic control, CLI + Placebo and CLI +
SR-Nitrite in isolated LAD coronary arteries (Figure 10A). LAD coronary artery from the
CLI + SR-Nitrite demonstrated a significantly (p < 0.05) greater relaxation at 10^-6 and
10^-5 M SNP compared to CLI + Placebo. Maximum relaxation in response to SNP
shown in Figure 10B reveals significantly greater (60% increase) vasorelaxation of the
CLI + SR-Nitrite LAD compared to CLI + Placebo (p < 0.05). However, the relaxation
was impaired compared to the LAD from lean non-ischemic controls (Figure 10), with
the CLI + SR-Nitrite group have a 30% decrease in maximum relaxation (p < 0.05).

Substance P and bradykinin were studied to assess endothelial-dependent relaxation in
isolated LAD coronary arteries. Although lean non-ischemic coronary arteries
demonstrated dose-dependent response to substance P (Figure 11A) and bradykinin
(Figure 11B), the relaxation of CLI + Placebo and CLI + SR-Nitrite coronary arteries
was impaired. There was no significant difference in the maximum relaxation response
(% of contraction) between the CLI + Placebo and CLI + SR-Nitrite groups in response
to 10^-8 M substance P or 10^-5 M bradykinin (Figures 11C and 11D).

Discussion

MetS, with a rising prevalence worldwide, is associated with increases in the risk of
cardiovascular morbidity and mortality (32). In the present study we have provided
evidence that the obese OS is a clinically relevant model of MetS that exhibits profound coronary vascular dysfunction that appears to be mediated via reduction in NO bioavailability and increased oxidative stress.

The OS has a “thrifty genotype” that enables the storage of excess food when available and thereby promotes survival during times of famine. In agreement with previously reported findings (11, 35), we observed that six months of a high fat, high cholesterol diet resulted in central obesity, elevated blood glucose, increased cholesterol, and increased triglycerides compared to lean OS fed a standard diet. As defined by the Adult Treatment Panel III (ATP III) (28) our data clearly demonstrate a MetS phenotype in the obese OS fed a high calorie, atherogenic diet.

The combination of cardiovascular risk factors associated with MetS has a significant impact on vascular integrity with many MetS patients presenting vascular dysfunction in conduits and small arteries (18, 42). It has been shown in clinical studies with CAD patients (7, 19, 44) that virtually every cardiovascular disease risk factor identified to date promotes endothelial dysfunction and attenuates NO bioavailability. Thus leading us to postulate that link between MetS and vascular disease, specifically CAD, is mediated by loss of eNOS functionality and reduced endogenous NO production. NO derived from coronary arteries and from cardiac myocytes plays a significant role in vessel reactivity and cardiac myocyte homeostasis helping to protect against pathological insult (29). NO synthesis in the heart and vessels is regulated primarily by eNOS. The generation of NO by eNOS is mediated by multisite phosphorylation at serine^{1177} and threonine^{495} amino acids sites either enhancing or inhibiting eNOS generation of NO, respectively (4, 26, 34). Endothelial cells from MetS patients display
increases in phosphorylation at the threonine\(^{495}\) and decreases in NO production (1). Furthermore, Marchesi \textit{et al.} demonstrate eNOS uncoupling in New Zealand obese mice with MetS impairing vascular function (33). In the current study, we observed a marked reduction in phosphorylation at serine\(^{1177}\) and an increased in phosphorylation at threonine\(^{495}\) in the hearts of the obese OS compared to the lean OS. These findings were coupled with concomitant reductions in myocardial nitrite and coronary cGMP, confirming decreased NO bioavailability in the heart and signaling in the coronaries.

The loss of eNOS function and reduction in NO bioavailability in response to MetS may be due to several possible mechanisms including: excessive oxidative stress and/or increased levels of endogenous eNOS inhibitors. Oxidative stress as measured by urinary 8-epi-PGF\(_2\alpha\) correlates to an increasing body mass index (BMI) in humans (14, 15). The Framingham Study reported that both BMI and diabetes are associated with systemic oxidative stress (22). In the present study, we observed significant increases in protein carbonyl content, a marker of oxidative stress, in myocardial tissue from the obese OS compared to the lean OS. In Fischer rats fed a high fat, high-refined sugar diet, Roberts and colleagues report reductions in the expression of the powerful antioxidant enzymes GPx-1 and HO-2 in the aorta (41). Although we observed no statistical change in GPX-1, obese OS had significantly lower antioxidant expression of myocardial HO-1 mRNA compared to the lean OS. Interestingly, we observed an increase in myocardial SOD2 mRNA levels in the obese OS group, suggesting a possible compensatory response to increased oxidative stress resulting in increased levels of this antioxidant enzyme. The obese OS also had marked increases in plasma ADMA, an endogenous inhibitor of eNOS. Several investigators have reported
increases in ADMA levels in rats and humans with various risk factors of MetS including hyperglycemia (25) and hypercholesterolemia (3). Moreover, a number of cardiovascular diseases correlate with increases in circulating ADMA and oxidative stress (45, 46).

Previous studies from our lab show that daily sodium nitrite supplementation enhances myocardial nitrite levels in murine cardiac disease models of NO deficiency (2, 6). Nitrite therapy reverses age-induced vascular endothelial dysfunction in mice (43). Because there is a high prevalence of MetS patients that present with clinical manifestations of atherosclerotic vascular disease, specifically PAD (17), we developed a model that would represent this high-risk population. Similar to CAD, PAD patients exhibit impaired NO synthesis (3) and endothelial dysfunction (5, 16). The vascular dysfunctions associated with PAD increase the risk of vascular complications in other area, including the coronary circulation (13). Since endogenous nitrite has been shown to elicit endocrine effects and mediate cytoprotection in remote organs (12), we hypothesized that chronic administration of sustained release nitrite would attenuate myocardial oxidative stress and improve coronary vascular reactivity in the setting of MetS and CLI.

In our current study, obese OS were treated daily with a SR-Nitrite formulation (80 mg p.o. b.i.d.) starting at 14 days following the onset of CLI injury. SR-Nitrite restored myocardial phosphorylation of the eNOS serine$^{1177}$ active site in the OS + SR-Nitrite group resulting in increased NO bioavailability in the heart. We determined that the dosage of SR-Nitrite that was employed in the current study did not reduce systemic blood pressure suggesting a favorable safety profile and possible clinical utility for peripheral vascular and other cardiovascular diseases.
Under certain physiological conditions NO is a potent scavenger of superoxide anion that modulates redox balance in the circulation and tissues. Thus, the balance between NO formation and superoxide is critical for cardiovascular homeostasis (8). In the current study, SR-Nitrite treatment attenuated MetS-induced increases in oxidative stress as the CLI + SR-Nitrite group exhibited significantly lower levels of the oxidative stress markers MDA and protein carbonyl. SR-Nitrite also resulted in significant elevations of GPx-1 and catalase. These effects may have occurred by the scavenging of the $O_2^-$ by the nitrite-derived NO.

Previous studies have evaluated vascular reactivity in the obese OS model. Payne et al. demonstrated that coronary arteries obtained from obese OS were associated with endothelial dysfunction, which was exacerbated in the presence of perivascular adipose tissue (39). In the present study, LAD coronary arteries obtained from the obese OS had attenuated contraction to KCl and PGF$_2$$\alpha$ compared to the York non-ischemic lean controls with no significant difference between the CLI + SR-Nitrite and CLI + Placebo. Similar to our findings, Owen et al. concluded that obesity augments calcium cycling and smooth muscle vasoconstriction after observing an attenuated contraction in obese OS coronaries compared to lean controls (38). Utilizing vasodilators that induce relaxation through endothelial-independent (SNP) and dependent (substance P and bradykinin) mechanisms we examined the vascular reactivity of LAD coronary arteries obtained from CLI + SR-Nitrite, CLI + Placebo, and non-ischemic lean controls pigs. PGF$_2$$\alpha$-induced pre-contracted LAD coronary arteries displayed impaired relaxation in response to SNP, substance P and bradykinin in the obese OS pigs compared to the
non-ischemic lean controls clearly indicating that vasodilatory properties of the vessel
are impaired in the setting of obesity and MetS. In the obese OS, SR-Nitrite therapy
improved relaxation in response to SNP indicating the partial restoration of smooth
muscle dependent vasorelaxation. However, we did not observe improved
vasoreactivity in response to an endothelial dependent vasorelaxation (i.e. substance P
or bradykinin) mechanism, signifying that the combination of MetS and CLI induces
endothelial dysfunction. Although chronic nitrite treatment has been shown to reverse
age – dependent endothelial dysfunction (43), we did not observe improvements in
endothelial dependent vasorelaxation. We believe that the primary reason for the
difference between the two experimental studies is that the obese Ossabaw Swine
subjected to critical limb ischemia represents a significantly more severe form of
cardiovascular disease as compared to aging alone. The obese Ossabaw Swine exhibit
severe hyperlipidemia, coupled with coronary artery atherosclerotic lesions and
therefore restoration of coronary endothelial-dependent vascular reactivity may not be
possible in these animals in this 35 day treatment protocol. Longer durations of SR-
Nitrite therapy may be required to partially restore endothelial-dependent coronary
vascular reactivity.

In conclusion, our findings demonstrate that OS fed a high fat, high cholesterol diet
results in a MetS phenotype that has significant impact on NO bioavailability by
impairing eNOS function in the heart. Concomitant with reduced NO bioavailability we
observed a significant increase in myocardial oxidative stress in the obese Ossabaw
swine. SR-Nitrite therapy restored cardiac eNOS function in the setting of severe CLI
via enhancing myocardial nitrite levels and attenuating oxidative stress. Although CLI in
the setting of MetS was associated with marked oxidative stress and profound endothelial dysfunction, SR-Nitrite therapy improved \textit{ex vivo} vascular function of coronary arteries via direct effects on the coronary vascular smooth muscle. SR-Nitrite therapy may prove beneficial for MetS patients with preexisting chronic cardiovascular disease states such as CLI that are characterized by upregulation of oxidative stress and vascular dysfunction.

**New & Noteworthy**

In a clinically relevant porcine model of metabolic syndrome and peripheral vascular disease, treatment with a novel sustained release Nitrite formulation restored cardiac eNOS enhancing myocardial nitrite levels, reduced oxidative stress, and improved \textit{ex vivo} coronary vascular function via endothelial-independent vasodilation mechanism in the obese Ossabaw Swine.
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Disclosures

D.J.L. is on the scientific advisory board of TheraVasc, Inc. and serves as an unpaid consultant. TheraVasc is currently developing sodium nitrite for the treatment of cardiovascular diseases. D.J.L. is a participant of a pending U.S. patent filed on 14 October 2003 (patent no. 60/511244) regarding the use of sodium nitrite in cardiovascular disease. D.J.L. is a participant of a pending U.S. patent filed on 15 November 2007 (patent no. 61/003150) regarding the use of nitrite salts in chronic ischemia.
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Figure Legends

Figure 1. Ossabaw swine fed a high fat, high cholesterol diet exhibit a metabolic syndrome phenotype. (A) Body weight, (B) plasma glucose, (C) insulin and (D) alanine transaminase (ALT). Values are mean ± SEM. Circles inside bars denotes the n per group. OS, Ossabaw swine.

Figure 2. Obese OS swine display dyslipidemia following a high fat high cholesterol diet. (A) Cholesterol, (B) triglycerides, (C) low density lipoproteins (LDL) cholesterol, (D) high density lipoprotein (HDL) cholesterol, and (E) very low-density lipoprotein (VLDL) cholesterol. Values are mean ± SEM. Circles inside bars denotes the n per group. OS, Ossabaw swine.

Figure 3. Metabolic syndrome decreases phosphorylation of eNOS at Ser^{1177} and increases phosphorylation of eNOS at Thr^{495}. These alterations in eNOS phosphorylation result in profound inhibition of eNOS and reduced myocardial NO bioavailability. (A) Representative immunoblots of cardiac protein expression of phosphorylated eNOS^{Ser^{1177}}, eNOS^{Thr^{495}}, and total eNOS. Densitometry analysis of (B) total eNOS, (C) phosphorylated eNOS^{Ser^{1177}}, and (D) phosphorylated eNOS^{Thr^{495}}. (E) Cardiac nitrite levels in lean and obese swine. (F) Coronary artery cGMP levels. Values are mean ± SEM. Circles inside bars denotes the n per group. OS, Ossabaw swine.
Figure 4. Metabolic syndrome increases oxidative stress in the heart. Myocardial (A) malondialdehyde (MDA) levels (nmol/gram of protein) and (B) carbonyl protein cardiac levels in lean OS and obese OS. (C) Plasma levels (µM) of asymmetric dimethylarginine (ADMA). Values are mean ± SEM. Circles inside bars denotes the n per group. OS, Ossabaw swine.

Figure 5. Metabolic syndrome alters the expression of antioxidant enzymes in the heart. (A) mRNA levels of glutathione peroxidase 1 (GPx-1), (B) mRNA levels of catalase, (C) mRNA levels of superoxide dismutase 2 (SOD 2), and (D) mRNA levels of hemeoxygenase 1 (HO-1) in myocardial tissue from lean OS and obese OS. Values are mean ± SEM. Circles inside bars denotes the n per group. OS, Ossabaw swine.

Figure 6. Experimental protocol for experiments of critical limb ischemia (CLI) in the obese Ossabaw swine. (A) Obese OS were subjected to critical limb ischemia (CLI) for 35 days. SR-Nitrite (80 mg p.o. b.i.d) or placebo therapy was initiated at 14 days following CLI injury. At day 35 following CLI, myocardial tissues were collected to evaluate endothelial nitric oxide synthase (eNOS) functionality, nitric oxide bioavailability, and oxidative stress following CLI. In addition, epicardial coronary artery segments were harvested and in vitro coronary vascular reactivity was evaluated. We also investigated the effect of CLI in the obese OS on systemic blood pressures. (B) AM and PM averages of mean arterial blood pressure (MABP). Values are mean ± SEM. (*) p < 0.05 versus CLI + Placebo) OS, Ossabaw swine.
**Figure 7.** SR-Nitrite therapy induces eNOS<sub>Ser1177</sub> active site phosphorylation and increases NO levels in the heart in the obese OS following CLI. (A) Representative Immunoblots of cardiac protein expression of phosphorylated eNOS<sub>Ser1177</sub>, eNOS<sub>Thr495</sub>, and total eNOS. Densitometry analysis of (B) total eNOS, (C) phosphorylated eNOS<sub>Ser1177</sub>, and (D) phosphorylated eNOS<sub>Thr495</sub>. (E) Cardiac nitrite levels in CLI + Placebo and CLI + SR-Nitrite. Values are mean ± SEM. Circles inside bars denotes the n per group. OS, Ossabaw Swine. CLI, critical limb ischemia.

**Figure 8.** Nitrite therapy with sustained release SR-Nitrite significantly reduced myocardial oxidative stress and increased antioxidant enzyme mRNA levels in the obese OS following critical limb ischemia (CLI). (A) Malondialdehyde (MDA), (B) carbonyl protein, (C) glutathione peroxidase 1 (GPx-1), (D) superoxide dismutase 1 (SOD1), (E) catalase, and (F) superoxide dismutase 2 (SOD2) mRNA levels in the hearts of CLI + Placebo and CLI + SR-Nitrite treated pigs. Values are mean ± SEM. Circles inside bars denotes the n per group. OS, Ossabaw swine. CLI, critical limb ischemia

**Figure 9.** Obese OS subjected to critical limb ischemia (CLI) exhibit impaired coronary vascular contraction as compared to non-ischemic, lean Yorkshire control animals. Left anterior descending coronary artery vasoconstriction with potassium chloride (KCl) at (A) 40 mM and (B) 100 mM or (C) prostaglandin F2α (PGF<sub>2α</sub>) at 30mM. Values are mean ± SEM. Circles inside bars denotes the n per group. OS, Ossabaw Swine.
Figure 10. SR-Nitrite therapy improves coronary vascular reactivity by an NO-dependent mechanism in the setting of metabolic syndrome and CLI. Freshly isolated coronary arteries were pre-contracted with prostaglandin PGF$_{2\alpha}$. (A) Dose-dependent relaxation (% of relaxation) of the left anterior descending-coronary artery in response to sodium nitroprusside (SNP). (B) Maximum relaxation at $10^{-5}$ M SNP. Values are mean ± SEM. Circles inside bars denotes the n per group. (*p < 0.05 versus CLI + Placebo) OS, Ossabaw Swine; CLI, critical limb ischemia.

Figure 11. SR-Nitrite therapy had no effect on coronary vascular reactivity to endothelium-dependent vasodilators. Freshly isolated coronary arteries were pre-contracted with prostaglandin PGF$_{2\alpha}$. Dose-dependent relaxation (% of relaxation) of the left anterior descending-coronary artery in response to (A) substance P and (B) bradykinin. Maximum relaxation at (C) $10^{-8}$ M substance P and (D) $10^{-5}$ M bradykinin. Values are mean ± SEM. Circles inside bars denotes the n per group.
Figure 1

(A) Body Weight (Kg) - p < 0.01

(B) Glucose (mg/dL) - p < 0.05

(C) Insulin (IU/mL) - p = NS

(D) ALT (U/L) - p < 0.05
Figure 2

(A) Cholesterol (mg/dL) with p < 0.0001

(B) Triglyceride (mg/dL) with p < 0.05

(C) LDL Cholesterol (mg/dL) with p < 0.01

(D) HDL Cholesterol (mg/dL) with p = NS

(E) VLDL Cholesterol (mg/dL) with p < 0.01
Figure 3

A. Western blot showing the expression of eNOS-P_Ser1177, eNOS-P_Thr495, Total eNOS, and α-Tubulin in Lean OS and Obese OS.

B. Bar graph showing the relative intensity of Total eNOS/α-Tubulin in Lean OS and Obese OS. The p-value is NS.

C. Bar graph showing the relative intensity of eNOS-P_Ser1177/Total eNOS in Lean OS and Obese OS. The p-value is <0.01.

D. Bar graph showing the relative intensity of eNOS-P_Thr495/Total eNOS in Lean OS and Obese OS. The p-value is <0.05.

E. Bar graph showing the myocardial nitrite concentration (µM/mg of protein) in Lean OS and Obese OS. The p-value is <0.01.

F. Bar graph showing the coronary artery cGMP (pmol/g) in Lean OS and Obese OS. The p-value is <0.05.
Figure 4

(A) Myocardial MDA (nmol/mg of protein) for Lean OS and Obese OS with p = NS.

(B) Myocardial Carbonyl (nmol/mg protein) for Lean OS and Obese OS with p < 0.05.

(C) Plasma ADMA (µM/L) for Lean OS and Obese OS with p < 0.05.
Figure 5

A. Myocardial mRNA GPx-1

B. Myocardial mRNA Catalase

C. Myocardial mRNA SOD2

D. Myocardial mRNA HO-1

- Lean OS
- Obese OS

Significance levels:
- p < 0.05
- p = NS
- p < 0.01
Critical Limb Ischemia in Obese Ossabaw Swine (OS)

Experimental Protocol

- Myocardial Tissue Collection
  - Molecular Analyses (Western blots, PCR)
  - Nitrite and RSNO Measurements
  - Biochemical Analysis
- Coronary Vascular Reactivity

Figure 6

Critical Limb Ischemia (CLI)

- DSI Radiotelemetry-Blood Pressure
- SR-Nitrite or Placebo (80 mg p.o. b.i.d)

Baseline

Time (Days)
Figure 7

**A**

- eNOS
- eNOS-P^Ser1177^ 140 kDa
- eNOS-P^Thr495^ 140 kDa
- GAPDH 37 kDa

**B**

- eNOS / GAPDH (Relative Intensity)

**C**

- eNOS-P^Ser1177^ (Relative Intensity)

**D**

- eNOS-P^Thr495^ (Relative Intensity)

**E**

- Myocardial Nitrite (nmol/mg of protein)

CLI + Placebo

CLI + SR-Nitrite

p < 0.05

p = NS

 CLI + Placebo

 CLI + SR-Nitrite

 CLI + Placebo

 CLI + SR-Nitrite

 CLI + Placebo

 CLI + SR-Nitrite

 CLI + Placebo

 CLI + SR-Nitrite
Figure 9

A) KCL 40 mM

Contraction (grams)

Non-ischemic Control
CLI + Placebo
CLI + SR-Nitrite

p = NS

B) KCL 100 mM

Contraction (grams)

Non-ischemic Control
CLI + Placebo
CLI + SR-Nitrite

p = NS

C) PGF\(_{2\alpha}\)

Contraction (grams)

Non-ischemic Control
CLI + Placebo
CLI + SR-Nitrite

p = NS
Figure 10

(A) Graph showing the percentage relaxation of Log Sodium Nitroprusside [M].

- Non-ischemic Control (n = 2)
- CLI + Placebo (n = 6)
- CLI + SR-Nitrite (n=5)

(B) Bar chart showing maximum relaxation (%).

- Non-ischemic Control
- CLI + Placebo (p < 0.05)
- CLI + SR-Nitrite
Figure 11

(A) Log Substance P [M] % Relaxation

- Non-ischemic Control (n = 7)
- CLI + Placebo (n = 6)
- CLI + SR-Nitrite (n = 4)

(B) Log Bradykinin [M] % Relaxation

- Non-ischemic Control (n = 2)
- CLI + Placebo (n = 6)
- CLI + SR-Nitrite (n = 5)

(C) Maximum Relaxation (%)

- Non-ischemic Control
- CLI + Placebo
- CLI + SR-Nitrite

(D) Maximum Relaxation (%)

- Non-ischemic Control
- CLI + Placebo
- CLI + SR-Nitrite

p = NS