Sustained release nitrite therapy results in myocardial protection in a porcine model of metabolic syndrome with peripheral vascular disease

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Bradley JM, Islam KN, Polhemus DJ, Donnarumma E, Brewster LP, Tao YX, Goodchild TT, Lefer DJ. Sustained release nitrite therapy results in myocardial protection in a porcine model of metabolic syndrome with peripheral vascular disease. Am J Physiol Heart Circ Physiol 309: H305–H317, 2015. First published May 8, 2015; doi:10.1152/ajpheart.00163.2015.—Metabolic syndrome (MetS) reduces endothelial nitric oxide (NO) bioavailability and exacerbates vascular dysfunction in patients with preexisting 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 NO synthase (eNOS) regulation in the obese Ossabaw swine (OS) model and to examine the effects of a novel, sustained-release formulation of sodium nitrite (SR-nitrite) on coronary vascular reactivity and myocardial redox status in obese OS subjected to critical limb ischemia (CLI). After 6 mo 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 a significant reduction in antioxidant enzymes. The efficacy of SR-nitrite therapy was examined in obese OS subjected to CLI. After 3 wk of treatment, SR-nitrite (80 mg·kg−1·day−1 bid po) 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 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 with placebo-treated animals. In 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.

Ossabaw swine; endothelial dysfunction; atherosclerosis; nitric oxide; sustained-release sodium nitrite

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 endothelial nitric oxide synthase, enhancing myocardial nitrite levels, reduced oxidative stress, and improved ex vivo coronary vascular function via endothelium-independent vasodilation mechanism in obese Ossabaw swine.

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 increased risk for the development of cardiovascular disease. In patients with preexisting 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 loss of endothelial function characterized by dysfunction of endothelial nitric oxide (NO) synthase (eNOS) and reduced 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 physiological 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). 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 (OS) were subjected to CLI for 35 days and treated with SR-nitrite starting at day 14. In this study, we failed to observe significant improvements in
hindlimb perfusion, revascularization, or perfusion pressures. However, we did observe significant increases in NO bioavailability in the plasma and ischemic skeletal muscle at 35 days post-CLI injury coupled with reductions in oxidative stress and induction in proangiogenic 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 present study were twofold. 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 OS model). Subsequently, we evaluated the effects of SR-nitrite on blood pressure, myocardial oxidative stress, NO bioavailability, and coronary artery vasoactivity in obese OS subjected to CLI.

Methods

Animals. Male and female OS (6–8 mo of age) obtained from Indiana University and female Yorkshire (3 mo of age) obtained from Palmetto Research Swine (Reevesville, SC) were handled in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This protocol was approved by the Institutional Animal Care and Use Committee of Emory University. Animals received placebo (n = 6) or 80 mg SR-nitrite (n = 7) by mouth (po) twice daily (bid). Treatment was initiated at 14 days after the induction of CLI and continued for 21 days.

DSI radiotelemetry implantation. Before the induction of CLI, obese OS (n = 8) underwent surgical implantation of an arterial blood pressure (BP) radiotelemeter (TA11PA-D70, Data Sciences, St Paul, MN) to assess the extent of hypertension in obese OS. 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) 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 BP (MAPs) during this sample period were computed and stored for offline analysis.

Euthanasia and tissue collection. After 35 days of CLI, 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 ~5 min, and animals were then euthanized while under deep inhalant anesthesia (isoflurane 5%). The heart and blood were collected.

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

CLI model. Castrated male obese OS (6–8 mo of age) subjected to CLI investigated in the present 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 O2. Preoperative analgesics buprenorphine (0.15 mg for each pig) was given along with perioperative antibiotics [cefaclor (1,000 mg)]. The postoperative analgesics carprofen (10 mg) and buprenorphine (0.15–0.3 mg) were given immediately after the procedure. Postoperative day 1, pigs received the additional analgesics buprenorphine (0.3 mg) in combination with carprofen (100 mg). All pigs were monitored during the procedure for heart rate, respiration, O2 saturation, end-tidal CO2, fluid volume, and body temperature. Activated clotting time was measured with a Hemachron after the arterial sheath was placed and heparin was given.

Using sterile conditions and under fluoroscopic guidance, an 8-Fr 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 s. A percutaneous guidewire (0.035 × 230 cm, Rosen Starter, Boston Scientific, Natick, MA) was positioned in the right external iliac artery. A 7-Fr delivery guide catheter (MACH 1 MP1, Boston Scientific) containing a self-expanding endoluminal endoprosthesis consisting of an expanded polytetrafluoroethylene (ePTFE) lining with an external nitinol stent (6 mm × 15 cm, Viabahn, W.L. Gore and Associates, Flagstaff, AZ) was advanced, positioned within the right external iliac artery, and deployed. Contrast arteriography was performed to evaluate vessel patency. After placement of the ePTFE-lined endoprosthesis, a self-expanding nitinol mesh occlusion device (8 × 7 mm, Amplatz Vascular Plug II, St. Jude Medical) 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, 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 CLI.

Sodium nitrite therapy. Sustained-release sodium nitrite tablets and placebo tablets were obtained from TheraVasc (Cleveland, OH). Animals received placebo (n = 6) or 80 mg SR-nitrite (n = 7) by mouth (po) twice daily (bid). Treatment was initiated at 14 days after the induction of CLI and continued for 21 days.

H306 NITRITE IMPROVES CORONARY VASCULAR REACTIVITY IN CRITICAL LIMB ISCHEMIA

NUTRIENTS IMPROVE CORONARY VASCULAR REACTIVITY IN CRITICAL LIMB ISCHEMIA

Coronary artery vasoreactivity in obese OS subjected to CLI.

Sodium nitrite improved coronary vascular reactivity in obese OS subjected to CLI. The SR-nitrite-induced increase in hemodynamic parameters and improvement in hindlimb perfusion in obese OS subjected to CLI.

Measurement of NO metabolites. Nitrite concentrations were quantified in ventricular tissue biopsies by ion chromatography (ENO2 Analyzer, Eicom, Kyoto, Japan).

cGMP radioimmunoassay. Coronary artery cGMP concentrations were quantified as previously described (23).

Western blot analysis. Ventricular tissue was used for Western blot analysis, which was performed as previously described (23). The following primary antibodies were used: VEGF, CD31 (Novus Biologicals, Littleton, CO), eNOS (Ser1177; Abcam, Cambridge, MA), eNOS, eNOS (Thr495; Cell Signaling, Danvers, MA), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, 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 previously described (23). The following primary antibodies were used: VEGF, CD31 (Novus Biologicals, Littleton, CO), eNOS (Ser1177; Abcam, Cambridge, MA), eNOS, eNOS (Thr495; Cell Signaling, Danvers, MA), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

Functional assessment of isolated coronary rings. Isolated coronary artery experiments were performed in obese OS and lean, nonischemic Yorkshire control pigs. CLI experiments involved obese OS that received either placebo or SR-nitrite (80 mg po bid). Obese OS vascular reactivity responses were compared with nonischemic lean Yorkshire responses. Coronary arteries from Yorkshire pigs (3 mo, Palmetto Farms) were used as a reference for a nonischemic control. Left anterior descending (LAD) arteries were dissected,
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
experiments. Coronary arteries were placed under 0.5 g of tension for
90–120 min to obtain a stable and optimal baseline passive tension.
Arteries were stimulated initially with 40 mM and then 100 mM KCl
\((n = 2, 6 \text{ nonischemic control, } n = 6 \text{ CLI + placebo, and } n = 6 \text{ CLI + SR-nitrite} )\) to assess vessel viability and contractile responsiveness.

After a series of washes, rings were precontracted with PGF2α (30
μM). At peak constriction, vascular function was assessed by the
addition of graded concentrations of sodium nitroprusside (SNP;
\(10^{-9} -10^{-5} \text{ M, } n = 2 \text{ nonischemic control, } n = 6 \text{ CLI + placebo, and } n = 5 \text{ CLI + SR-nitrite} )
), bradykinin \((10^{-9} -10^{-5} \text{ M, } n = 2 \text{ nonischemic control, } n = 6 \text{ CLI + placebo, and } n = 5 \text{ CLI + SR-nitrite} ), or
substance P \((10^{-11} -10^{-8} \text{ M, } n = 2 \text{ nonischemic control, } n = 6 \text{ CLI + placebo, and } n = 4 \text{ CLI + SR-nitrite} ), to the tissue bath.

Statistical analysis. All data in the present study are expressed as
means ± SE. Differences in data between groups were compared
using Prism 6 (GraphPad Software). A Student’s unpaired, two-tailed
t-test was used when comparison was between two groups (i.e., lean
OS vs. obese OS or CLI + placebo vs. CLI + SR-nitrite). For
ambulatory MABP, two-way ANOVA was used to test the effects of
placebo and SR-nitrite therapy over time. For isometric tension
dose-dependent experiments, two-way ANOVA was used to test the
placebo and SR-nitrite therapy over time. For isometric tension
response. For maximal relaxation, one-way ANOVA was used to test
the effect of the treatment and the maximum dose of vasodilator on

RESULTS

The obese OS is a well-characterized model of central
obesity and MetS (11, 35). The data reported in the present
study represent data collected from two independent experi-
mental studies. Initial experiments evaluated the cardiovascu-
lar and MetS phenotype in obese OS compared with lean OS.
The results from the first study are shown in Figs. 1–5. The second study reported in the present report is a subset of a
study in which we investigated the efficacy of a novel extend-
ed-release formulation of nitrite (i.e., SR-nitrite) in a CLI
model (40). The present study is focused on the effects of SR-nitrite on myocardial oxidative stress, coronary vascular
reactivity, and systemic hemodynamics in obese OS subjected
to CLI. These results are shown in Figs. 6–11.

Characterization of MetS in obese OS. After 6 mo of a
high-fat, high-cholesterol diet, obese OS exhibited character-
istics of a MetS phenotype (Figs. 1 and 2) compared with lean
controls. Obese OS displayed a significant \((P < 0.01)\)
increase in body weight (Fig. 1A) compared with lean OS \((73.0 ± 5.0
vs. 40.0 ± 2.0 \text{ kg})\). There was no significant difference in
plasma insulin levels (Fig. 1C); however, blood glucose (Fig.
1B) was elevated \((P < 0.05)\) in obese OS \((80.2 ± 4.0 \text{ mg/dl})\)
compared with lean OS \((67.4 ± 4.6 \text{ mg/dl})\). Obese OS exhib-
it twofold higher circulating levels \((53.5 ± 9.1 \text{ U/l})\) of
alanine aminotransferase (ALT; \(P < 0.05\); Fig. 1D) compared with
lean OS \((24.3 ± 1.2 \text{ U/l})\). Lee et al. (24) reported that after
6 mo of feeding with a very similar high-fat, high-cholesterol
diet, OS displayed abnormal fatty liver histology that is similar
to human nonalcoholic steatohepatitis. Therefore, we suspect
that the increases in ALT that we observed in the present study
are related to steatohepatitis.

The atherogenic diet resulted in significant dyslipidemia in
obese OS, as shown in Fig. 2. Circulating cholesterol levels
were significantly \((P < 0.0001)\) elevated in obese OS \((508.5 ± 78.9 \text{ mg/dl})\)
compared with lean OS \((66.4 ± 2.7 \text{ mg/dl})\). Similarly, obese OS exhibited significant \((P < 0.05)\) elevations

![Fig. 1. Ossabaw swine (OS) fed a high-fat, high-cholesterol diet exhibit a metabolic syndrome (MetS) phenotype. A: body weight. B: plasma glucose. C: insulin. D: alanine transaminase (ALT). Values are means ± SE; numbers inside bars are numbers of animals per group.](http://ajpheart.physiology.org/)
Fig. 2. Obese OS display dyslipidemia after a high-fat, high-cholesterol diet. A: cholesterol. B: triglycerides. C: LDL-cholesterol. D: HDL-cholesterol. E: VLDL-cholesterol. Values are means ± SE; numbers inside bars are numbers of animals per group.

in plasma triglycerides (Fig. 2B) versus lean OS (59.1 ± 13.4 vs. 25.4 ± 2.7 mg/dl). Although there was no significant difference between lean OS and obese OS in terms of HDL-cholesterol (Fig. 2D), circulating LDL-cholesterol (Fig. 2C) was increased (P < 0.01) in obese OS (290.0 ± 51.0 mg/dl) compared with lean OS (26.6 ± 2.1 mg/dl). VLDL-cholesterol levels (Fig. 2E) demonstrated significantly (P < 0.01) higher VLDL in obese OS (26.0 ± 4.0 mg/dl) versus lean OS (10.5 ± 0.2 mg/dl).

OS, 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 has been well appreciated that phosphorylation of Ser1177 on eNOS activates the enzyme to increase NO production, whereas phosphorylation of Thr495 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 (Fig. 3B). Obesity and MetS resulted in a significant reduction in phosphorylation of eNOS at the primary activation site, Ser1177 (Fig. 3C), in obese OS hearts compared with 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, Thr495 (2.6 ± 0.2 vs. 1.1 ± 0.3 RI, obese OS vs. lean OS, respectively, P < 0.05; Fig. 3D). Myocardial tissue nitrite levels were measured as a surrogate index of NO bioavailability in the heart (Fig. 3E). Obese OS displayed reduced cardiac nitrite levels compared with 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 cGMP (Fig. 3F) was significantly (P < 0.05) decreased in coronary arteries of obese OS (2.0 ± 2.0 pmol/g) compared with lean OS (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 obese OS may be mediated by several
mechanisms, such as excessive oxidative stress and/or increased levels of endogenous eNOS inhibitors. To determine the overall redox status in obese OS hearts, we measured the extent of oxidative stress in myocardial tissue samples using MDA and carbonyl as overall indexes of oxidative stress. These data are shown in Fig. 4. Cardiac MDA levels were similar between obese OS and lean OS (Fig. 4A). Obese OS (3.1 ± 0.5 nmol/protein) did exhibit significantly (P < 0.05) elevated myocardial carbonyl protein levels (Fig. 4B) compared with lean OS (2.1 ± 0.2 nmol/protein). 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 (Fig. 4C) levels were significantly (P < 0.05) higher in obese OS (2.3 ± 0.1 μM/l) compared with lean OS (0.7 ± 0.1 arbitrary units).

We also measured mRNA levels of critical antioxidant enzymes in myocardial tissue from lean and obese OS (Fig. 5) to further evaluate the extent of oxidative stress. Glutathione peroxidase (GPx)-1 (Fig. 5A) and catalase (Fig. 5B) mRNA levels were not statistically different between the study groups; however, obese OS had a significant reduction in hemeoxygenase (HO)-1 (Fig. 5D) mRNA in the heart compared with lean OS (2.1 ± 0.3 vs. 5.8 ± 0.9 arbitrary units, P < 0.01). In contrast, quantitative PCR analysis of SOD2 mRNA (P < 0.05; Fig. 5C) revealed a marked increase in obese OS hearts (1.2 ± 0.1 arbitrary units) compared with lean OS (0.7 ± 0.1 arbitrary units).

Investigation of hypertension, myocardial redox status, and coronary vascular reactivity in obese OS after nitrite therapy. By using a severe model of peripheral vascular disease, we sought to investigate the effects of MetS in the setting of CLI on hypertension, 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 shown in Figs. 6–11.

Hypertension in obese OS and effects of SR-nitrite. Using obese OS, CLI injury was surgically induced in the right external iliac artery and sustained for 35 days, as shown in the experimental protocol in Fig. 6. SR-nitrite (80 mg po bid) or placebo therapy was initiated on day 14 after CLI surgery. To ensure that SR-nitrite therapy did not induce systemic hypotension, MABP was assessed by radiotelemetry. Ambulatory

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Fig. 3. MetS decreases phosphorylation of endothelial nitric oxide (NO) synthase (eNOS) at Ser1177 and increases phosphorylation of eNOS at Thr695. 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 (Ser1177), eNOS (Thr695), and total eNOS. B–D: densitometry analysis of total eNOS (B), phosphorylated eNOS (Ser1177; C), and phosphorylated eNOS (Thr695; D). E: cardiac nitrite levels in lean and obese OS. F: coronary artery cGMP levels. Values are means ± SE; numbers inside bars are numbers of animals per group. NS, not significant.
AM and PM averages of MABP are shown in Fig. 6B and revealed that obese OS exhibited 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 BP related to increased circulating NO levels and profound vasodilation. SR-nitrite therapy at the dosage investigated in the present study failed to exert a significant effect on MABP compared
SR-nitrite therapy reduced oxidative stress in obese OS after CLI. We next evaluated the effects of chronic SR-nitrite treatment on myocardial oxidative stress in obese OS after CLI injury (Fig. 8). Cardiac levels of MDA (Fig. 8A) were significantly ($P < 0.05$) reduced in the CLI + SR-nitrite-treated group (3.3 ± 0.2 nmol/mg protein) compared with the CLI + placebo-treated group (4.1 ± 0.3 nmol/mg protein). Similarly, carbonyl protein levels (Fig. 8B) in CLI + SR-nitrite-treated hearts (20.5 ± 0.8 nmol/mg protein) were significantly ($P < 0.05$) attenuated compared with CLI + placebo-treated hearts (25.9 ± 1.5 nmol/mg protein). SR-nitrite therapy (23.7 ± 2.9 arbitrary units) increased myocardial GPx-1 ($P < 0.05$; Fig. 8C) levels in the CLI + SR-nitrite-treated group compared with the CLI + placebo-treated control group (14.2 ± 1.7 arbitrary units). Although there was a trend toward an increase, there was no difference in myocardial catalase, SOD1, or SOD2 (Fig. 8, D–F) between CLI + SR-nitrite- and CLI + placebo-treated groups ($P$ = not significant 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 the present study was to examine the effects of chronic administration of SR-nitrite on coronary vascular reactivity in vitro. LAD arteries were removed from lean nonsmoking control pigs (Yorkshire domestic farm pigs) and from CLI-treated obese OS that received placebo or SR-nitrite for 21 days. These data are shown in Figs. 9–11. The contraction response (Fig. 9) to KCl and PGF2α demonstrated differences between nonsmoking control swine and CLI-treated obese OS. Using lean nonsmoking Yorkshire pigs as a reference, we observed a 49% reduction in contraction of CLI-treated obese OS coronary arteries at concentrations of 40 mM (Fig. 9A) and 100 mM (Fig. 9B) KCl compared with nonsmoking control swine. The LAD contraction response to PGF2α (Fig. 9C) was attenuated by 72% in CLI-treated obese OS compared with nonsmoking controls ($P < 0.001$). There was no significant difference between the CLI + placebo-treated group compared with the CLI + SR-nitrite-treated group ($P$ = not significant between groups).

Administration of SNP resulted in a dose-dependent relaxation from PGF2α-induced contraction in nonsmoking control, CLI + placebo-treated, and CLI + SR-nitrite-treated isolated LAD coronary arteries (Fig. 10A). LAD coronary arteries from the CLI + SR-nitrite-treated group demonstrated a significantly ($P < 0.05$) greater relaxation at $10^{-6}$ and $10^{-5}$ M SNP compared with the CLI + placebo-treated group. Maximum relaxation in response to SNP (Fig. 10B) revealed a significantly greater (60% increase) vasorelaxation of CLI + SR-nitrite-treated LAD arteries compared with CLI + placebo-treated LAD arteries ($P < 0.05$). However, the relaxation was impaired compared with LAD arteries from lean nonsmoking controls (Fig. 10), with the CLI + SR-nitrite-treated group have a 30% decrease in maximum relaxation ($P < 0.05$).
Substance P and bradykinin were studied to assess endothelium-dependent relaxation in isolated LAD coronary arteries. Although lean nonischemic coronary arteries demonstrated a dose-dependent response to substance P (Fig. 11A) and bradykinin (Fig. 11B), the relaxation of CLI/placebo- and CLI/SR-nitrite-treated coronary arteries was impaired. There was no significant difference in the maximum relaxation response (percentage of contraction) between CLI/placebo- and CLI + SR-nitrite-treated groups in response to 10⁻⁸ M substance P or 10⁻⁵ M bradykinin (Fig. 11, C and D).

**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 6 mo of a high-fat, high-cholesterol diet resulted in central obesity, elevated blood glucose, increased cholesterol, and increased triglycerides compared with lean OS fed a standard diet. As defined by Adult Treatment Panel 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 in patients with coronary artery disease (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 the link between MetS and vascular disease, specifically coronary artery disease, is mediated by loss of eNOS functionality and reduced endogenous NO production. NO derived from coronary arteries and 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 Ser1177 and Thr495 amino acid sites either enhancing or inhibiting eNOS generation of NO, respectively (4, 26, 34). Endothelial cells from MetS patients display increases in phosphorylation at Thr495 and decreases in NO production (1). Furthermore, Marchesi et al. (33) have demonstrated eNOS uncoupling in New Zealand obese mice with MetS impairing vascular function. In the present study, we observed a marked reduction in phosphorylation at Ser1177 and an increase in phosphorylation at Thr495 in the hearts of obese OS compared with 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-PGF2α, correlates to an

Fig. 8. Nitrite therapy with sustained release SR-nitrite significantly reduced myocardial oxidative stress and increased antioxidant enzyme mRNA levels in obese OS after CLI. A–F: MDA (A), carbonyl protein (B), GPx-1 (C), SOD1 (D), catalase (E), and SOD2 (F) mRNA levels in the hearts of CLI + placebo- and CLI + SR-nitrite-treated pigs. Values are means ± SE; numbers inside bars are numbers of animals per group.
increasing body mass index in humans (14, 15). The Framingham study (22) reported that both body mass index and diabetes are associated with systemic oxidative stress. In the present study, we observed significant increases in protein carbonyl content, a marker of oxidative stress, in myocardial tissue from obese OS compared with lean OS. In Fischer rats fed a high-fat, high-refined sugar diet, Roberts and colleagues (41) reported reductions in the expression of the powerful antioxidant enzymes GPx-1 and HO-2 in the aorta. Although we observed no statistical change in GPx-1, obese OS had a significantly lower antioxidant expression of myocardial HO-1 mRNA compared with 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. 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 laboratory have shown 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 coronary artery disease, PAD patients exhibit impaired NO synthesis (3) and endothelial dysfunction (5, 16).

Fig. 9. Obese OS subjected to CLI exhibit impaired coronary vascular contraction compared with nonischemic, lean Yorkshire control animals. A–C: left anterior descending coronary artery vasoconstriction with KCl at 40 mM (A) and 100 mM (B) or PGF$_{2\alpha}$ at 30 mM (C). Values are means ± SE; numbers inside bars are numbers of animals per group.

Fig. 10. SR-nitrite therapy improves coronary vascular reactivity by an NO-dependent mechanism in the setting of MetS and CLI. Freshly isolated coronary arteries were precontracted with PGF$_{2\alpha}$. A: dose-dependent relaxation (%relaxation) of the left anterior descending coronary artery in response to sodium nitroprusside. B: maximum relaxation at $10^{-5}$ M sodium nitroprusside. Values are means ± SE; numbers inside bars are numbers of animals per group. *$P < 0.05$ vs. the CLI + placebo-treated group.
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 SR-nitrite would attenuate myocardial oxidative stress and improve coronary vascular reactivity in the setting of MetS and CLI. In the present study, obese OS were treated daily with a SR-nitrite formulation (80 mg po bid) starting at 14 days after the onset of CLI injury. SR-nitrite restored myocardial phosphorylation of the eNOS Ser1177 active site in the SR-nitrite-treated OS group, resulting in increased NO bioavailability in the heart. We determined that the dosage of SR-nitrite that was used in the present study did not reduce systemic BP, 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 the redox balance in the circulation and tissues. Thus, the balance between NO formation and superoxide is critical for cardiovascular homeostasis (8). In the present study, SR-nitrite treatment attenuated MetS-induced increases in oxidative stress as the CLI + SR-nitrite-treated 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 superoxide anion by nitrite-derived NO.

Previous studies have evaluated vascular reactivity in the obese OS model. Payne et al. (39) demonstrated that coronary arteries obtained from obese OS were associated with endothelial dysfunction, which was exacerbated in the presence of perivascular adipose tissue. In the present study, LAD coronary arteries obtained from obese OS had attenuated contraction to KCl and PGF2α compared with Yorkshire nonischemic lean control swine with no significant difference between CLI + SR-nitrite- and CLI + placebo-treated groups. Similar to our findings, Owen et al. (38) concluded that obesity augments Ca2+ cycling and smooth muscle vasoconstriction after observing an attenuated contraction in obese OS coronary arteries compared with lean control arteries. Using vasodilators that induce relaxation through endothelium-independent (SNP) and endothelium-dependent (substance P and bradykinin) mechanisms, we examined the vascular reactivity of LAD coronary arteries obtained from CLI + SR-nitrite-treated pigs, CLI + placebo-treated pigs, and nonischemic lean control pigs. PGF2α-induced precontracted LAD coronary arteries displayed impaired relaxation in response to SNP, substance P, and bradykinin in obese OS compared with nonischemic lean control swine, clearly indicating that vasodilatory properties of the vessel are impaired in the setting of obesity and MetS. In 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 endothelium-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 endothelium-dependent vasorelaxation. We believe that the primary reason for the difference between the two experimental studies is that obese OS subjected to CLI represent a significantly more severe form of cardiovascular disease compared with aging alone. Obese OS exhibit severe hyperlipidemia coupled with coronary artery atherosclerotic lesions, and, therefore, restoration of coronary endothelium-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 endothelium-dependent coronary vascular reactivity.

In conclusion, our findings demonstrate that OS fed a high-fat, high-cholesterol diet result 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 obese OS. SR-nitrite therapy restored cardiac eNOS function in the setting of severe CLI by 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 ex vivo vascular function of coronary arteries via direct effects on 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 an upregulation of oxidative stress and vascular dysfunction.

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DISCLOSURES

D. J. Lefer is on the scientific advisory board of TheraVasc. TheraVasc is currently developing sodium nitrite for the treatment of cardiovascular diseases. D. J. Lefer is a participant of a pending United States patent filed on October 14, 2003 (patent no. 60/512,244), regarding the use of sodium nitrite in cardiovascular disease. D. J. Lefer is a participant of a pending United States patent filed on November 15, 2007 (patent no. 61/003150), regarding the use of nitrite salts in chronic ischemia.

AUTHOR CONTRIBUTIONS


REFERENCES


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