Chronic NOS inhibition accelerates NAFLD progression in an obese rat model

Ryan D. Sheldon,1,2 Jaume Padilla,2,3,4 Nathan T. Jenkins,8 M. Harold Laughlin,4,5,6 and R. Scott Rector1,2,7

1Research Service, Harry S Truman Memorial Veterans Affairs Hospital, Columbia, Missouri; 2Department of Nutrition and Exercise Physiology, University of Missouri, Columbia, Missouri; 3Department of Child Health, University of Missouri, Columbia, Missouri; 4Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri; 5Department of Biomedical Sciences, University of Missouri, Columbia, Missouri; 6Department of Medical Physiology and Pharmacology, University of Missouri, Columbia, Missouri; 7Department of Medicine-Gastroenterology and Hepatology, University of Missouri, Columbia, Missouri; and 8Department of Kinesiology, University of Georgia, Athens, Georgia

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Sheldon RD, Padilla J, Jenkins NT, Laughlin MH, Rector RS. Chronic NOS inhibition accelerates NAFLD progression in an obese rat model. Am J Physiol Gastrointest Liver Physiol 308: G540–G549, 2015. First published January 8, 2015; doi:10.1152/ajpgi.00247.2014.—The progression in nonalcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis is a serious health concern, but the underlying mechanisms remain unclear. We hypothesized that chronic inhibition of nitric oxide (NO) synthase (NOS) via Nω-nitro-l-arginine methyl ester (l-NAME) would intensify liver injury in a rat model of obesity, insulin resistance, and NAFLD. Obese Otsuka Long-Evans Tokushima fatty (OLETF) and lean Long-Evans Tokushima Otsuka (LET0) rats received control or l-NAME (65–70 mg·kg−1·day−1)-containing drinking water for 4 wk. l-NAME treatment significantly (P < 0.05) reduced serum NO metabolites and food intake in both groups. Remarkably, despite no increase in body weight, l-NAME treatment increased hepatic triacylglycerol content (+40%, P < 0.05) vs. control OLETF rats. This increase was associated with impaired (P < 0.05) hepatic mitochondrial state 3 respiration. Interestingly, the opposite effect was found in LET0 rats, where l-NAME increased (P < 0.05) hepatic mitochondrial state 3 respiration. In addition, l-NAME induced a shift toward proinflammatory M1 macrophage polarity, as indicated by elevated hepatic CD11c (P < 0.05) and IL-1β (P = 0.07) mRNA in OLETF rats and reduced expression of the anti-inflammatory M2 markers CD163 and CD206 (P < 0.05) in LET0 rats. Markers of total macrophage content (CD68 and F4/80) mRNA were unaffected by l-NAME in either group. In conclusion, systemic NOS inhibition in the obese OLETF rats reduced hepatic mitochondrial respiration, increased hepatic triacylglycerol accumulation, and increased hepatic inflammation. These findings suggest an important role for proper NO metabolism in the hepatic adaptation to obesity.

Nω-nitro-l-arginine methyl ester; obese Otsuka Long-Evans Tokushima fatty rat; nitric oxide; liver

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) comprises a spectrum of liver pathologies from steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis (31). In the United States, NAFLD prevalence doubled from 1988 to 2008 and currently accounts for ~75% of the national chronic liver disease burden (41). While hepatic steatosis is relatively benign, progression of the disease to NASH and fibrosis is associated with substantially increased health risk. In a large cohort of NAFLD patients, those with biopsy-confirmed NASH had a 16% liver-related mortality rate compared with 3% in the non-NASH patients during a 150-mo follow-up (36). Thus efforts to understand the mechanisms that contribute to advancing NAFLD severity could provide valuable insight into the development of therapeutic options.

Nitric oxide (NO) is ubiquitously produced via two constitutive NO synthase (NOS) isoforms, neuronal (nNOS) and endothelial (eNOS), and an inducible isoform (iNOS). The important physiological role of constitutive NO is well appreciated in the cardiovascular and nervous systems, where it is exerted potent vasodilatory and anti-inflammatory effects. Alternatively, iNOS is upregulated in inflammatory conditions and can produce excess NO, which can cause cellular damage. Limited evidence indicates that constitutive NO production can confer hepatoprotection by preventing lipopolysaccharide-induced necrosis (21) and oxidative damage (11) and maintain appropriate hepatic perfusion (33). In addition, NO can influence key processes involved in NAFLD progression, including mitochondrial function (3) and biogenesis (19), fatty acid oxidation (37), Kupffer cell polarization (38), and hepatic stellate cell fibrogenesis (40). However, there is a paucity of data regarding the role of hepatic NO as it relates to NAFLD severity that warrants attention.

The spontaneously hyperphagic Otsuka Long-Evans Tokushima fatty (OLETF) rat is a well-characterized model of obesity that develops NAFLD in a pattern similar to humans (30). Hepatic mitochondrial dysfunction, a key feature of NAFLD in this model, is present through all stages of the disease progression (30). Importantly, NO influences mitochondrial biogenesis (7) and electron transport chain (ETC) function (3). We recently reported an association between reduced hepatic eNOS activation status and NASH onset in OLETF rats (34). This finding led us to hypothesize in the current investigation that impaired constitutive NO production may contribute to NAFLD severity. If true, such a concept could have an important and rapid clinical impact, given the wide array of NO-based therapies (10).

To assess the role of constitutive NO production in NAFLD, we administered Nω-nitro-l-arginine methyl ester (l-NAME) for 4 wk in OLETF rats at an age when they have significant hepatic steatosis but lack features of NASH, including inflammation (and, therefore, increased iNOS expression). In addition, lean counterparts of the OLETF [Long-Evans Tokushima

Address for reprints and other correspondence: R. S. Rector, Research Service-Harry S Truman Memorial VA Hospital and Depts. of Medicine and Nutrition and Exercise Physiology, Univ. of Missouri-Columbia, Columbia, MO 65212 (e-mail: rectors@health.missouri.edu).
Otsuka (LETO)) rats were studied to identify specific obesity-dependent processes in the liver that are affected by NOS inhibition. We hypothesized that l-NAME-treated OLETF rats would demonstrate features of more advanced NAFLD/NASH, including mitochondrial dysfunction, hepatic inflammation, and fibrosis, compared with control OLETF rats.

**METHODS**

**Animal protocol.** OLETF and LETO rats were housed in a climate-controlled room with a 12:12-h light-dark cycle from 4 to 20 wk of age with ad libitum access to water and standard rat chow (Formulab Diet 5008, LabDiet, St. Louis, MO). At 16 wk of age, OLETF and LETO rats were randomly selected to receive control drinking water or drinking water containing l-NAME (60–70 mg·kg⁻¹·day⁻¹) for 4 wk. Body weight and food consumption were measured weekly. On the day they were euthanized, the animals were anesthetized at 0800 with pentobarbital sodium (1 mL/kg) after an overnight fast. Body composition was determined via dual-energy X-ray absorptiometry.

Blood was collected via cardiac puncture and stored at −80°C for later processing, and animals were euthanized via cardiectomy. The liver was rapidly removed, and portions consistently taken from the same anatomic location were placed in mitochondrial isolation buffer (see below), fixed in formalin, or snap-frozen in liquid nitrogen. All animal protocols were approved by the University of Missouri Animal Care and Use Committee.

**Liver morphology, triacylglycerol, and total nitrate content.** Formalin-fixed tissue was mounted in paraffin, serially sliced, and stained with hematoxylin and eosin. Biochemical quantification of intrahepatic triacylglycerol (TAG) content was measured as described by our group previously (28). Content of total hepatic nitrate and nitrite (NOX), stable oxidation products of NO, was assessed in whole liver homogenate via a commercially available kit (product no. 780001, Cayman, Ann Arbor, MI).

**Blood assays.** Serum insulin, glucose, TAG, free fatty acid (FFA), cholesterol, and plasma concentrations of NOX were determined as reported previously (23).

**Hepatic mitochondrial isolation.** Mitochondria were isolated from freshly harvested liver as previously described (8). Total protein concentration of each isolated mitochondrial preparation was measured via bicinchoninic acid reaction and used to normalize functional values to protein loaded.

**Mitochondrial respiration and fatty acid oxidation.** Mitochondrial respiration was determined using the Oxygraph 2K (Oroboros Instruments, Innsbruck, Austria) as described previously (8). State 2 respiration was determined using the Oxygraph 2K (Oroboros Instruments, Innsbruck, Austria) as described previously (8). State 2 respiration was assessed by serial addition of ADP (25–125 μM) to promote oxidative phosphorylation (state 3, complex I). State 3 respiration was further assessed by addition of 10 mM succinate to stimulate complex II activity (state 3, complexes I and II). Finally, the uncoupling agent carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (250 nmol) was added to determine maximum uncoupled respiratory capacity. Fatty acid oxidation was determined in isolated mitochondria by addition of [1-¹⁴C]palmitate as described by our group previously (28).

**Mitochondrial phosphorylation-specific eNOS (p-eNOS; catalog no. 612393), and nNOS (catalog no. 610310) from BD Biosciences (San Jose, CA); iNOS (catalog no. ab3523), CD36 (catalog no. ab133625), cytochrome c oxidase (COX) subunit I (COX1; catalog no. ab14705), and OXPHOS mitochondrial profile (catalog no. ab110413) from Abcam (Cambridge, MA); and acetyl-CoA carboxylase (ACC; catalog no. 3662), S79 phosphorylation-specific ACC (catalog no. 3661), fatty acid synthase (FAS; catalog no. 3189), microsomal triglyceride transfer protein (MTTP; catalog no. ab186446), cytochrome c (catalog no. 4272), α-AMP-activated protein kinase (AMPK; catalog no. 2603), T172 phosphorylation-specific AMPK (p-AMPK; catalog no. 2531), Akt (catalog no. 9272), and S473 phosphorylation-specific Akt (catalog no. 9271) from Cell Signaling (Danvers, MA). Blots were analyzed via densitometric analysis (Image Lab Beta 3, Bio-Rad Laboratories, Burlingame, CA). Total protein was assessed with amido black (0.1%; Sigma) to control for differences in protein loading and transfer as previously described by our group (28).

**Gene expression.** RNA was extracted from homogenized liver tissue by a commercially available RNeasy Mini Kit (product no. 74104, Qiagen, Valencia, CA), and reverse transcriptase was used to synthesize cDNA. RNA and cDNA quality was determined by a spectrophotometer (Nanodrop 2000c, Thermo Scientific, Waltham, MA). Quantitative real-time PCR was completed with the ABI 7500 Fast Sequence Detection System (Applied Biosystems, Carlsbad, CA) using Fast SYBR Green Master Mix (Applied Biosystems). Primer pairs were obtained from Sigma (St. Louis, MO) and are listed in Table 1. Dissociation melt curves were analyzed to verify primer specificity. Transcript abundance of β-actin was not different among groups and was used as the reference gene to calculate expression levels of genes of interest using the comparative threshold (2⁻∆∆CT) method. All data are normalized to expression levels of the LETO control group.

**Enzyme assays.** Citrate synthase and β-hydroxacycl-CoA dehydrogenase activities were determined in freeze-fractured isolated hepatic mitochondria using the methods of Srere (35) and Bass et al. (2), respectively, and as described previously by our group (28). Superoxide dismutase (SOD) activity was determined in whole liver homogenate via a commercially available kit (product no. 706002, Cayman). Hepatic NOS activity was assessed in whole liver homogenate via a commercially available kit (product no. 781001, Cayman) according to the manufacturer’s instructions using [1-¹⁴C]arginine. Briefly, this assay assesses the catalytic conversion of [1-¹⁴C]arginine to [1-¹⁴C]citrulline in the presence of essential NOS cofactors. Snap-frozen liver samples were homogenized and incubated in reaction medium at 37°C for 15 min. To assess the relative contribution of Ca²⁺-dependent NOS (eNOS and nNOS), Ca²⁺ was omitted from the reaction medium and 5 mM EDTA was added to chelate residual Ca²⁺. Background counts were subtracted from each reading and were assessed by incubation of each sample without Ca²⁺ plus addition of 1 mM N-nitro-l-arginine (l-NNA), a nonspecific NOS inhibitor. By inference, the Ca²⁺-independent (i.e., iNOS) contribution to total hepatic NOS activity was considered to be the difference in counts between Ca²⁺-depleted and l-NNA-inhibited counts.

**Statistical analysis.** Statistical significance (P < 0.05) was assessed via one-way ANOVA for each measure using the open-access statistical software R (version 2.15.1). When a significant main effect was observed, Fisher’s least significant difference test was used for post hoc comparisons. Values are means ± SE of 8–10 observations per group.

**RESULTS**

Food intake, body composition, and blood characteristics for the animals are reported elsewhere (23) and summarized in Table 2. Four weeks of l-NAME treatment significantly reduced plasma NOX in LETO and OLETF rats. OLETF rats consumed more food, gained more weight, and had a higher percent body fat than LETO rats. l-NAME treatment in OLETF rats caused a significant reduction in weight gain, food intake, and percent body fat relative to the OLETF control group during the 4-wk treatment period. In addition, OLETF rats had elevated concentrations of plasma glucose and insulin, and l-NAME treatment in OLETF rats significantly...
Table 1. SYBR Green PCR primer pairs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Primers (5' → 3')</th>
<th>Reverse</th>
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<tr>
<td>α-SMA</td>
<td>TGAAGAGGAAAGACAGCCACAGG</td>
<td>CCAACATCAGCTCCGCTGTTG</td>
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<tr>
<td>β-Actin</td>
<td>CAGGACAGAGAGAGACATTCTC</td>
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<td>CD11c</td>
<td>CTGGTACATGGACGGACGAGA</td>
<td>AACTGTCGACCGTTGCTCCT</td>
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<td>CD68</td>
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<td>CAGGACAAGGGCTGCTGCTT</td>
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<td>CD206</td>
<td>GAGGACTCTGGCTGTGATAAGA</td>
<td>CAGGACGGGTGTTGAGTGTG</td>
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<tr>
<td>Col-1α</td>
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<td>CAGGACAGACTCAGGTAAC</td>
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<tr>
<td>F4/80</td>
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<td>HIF-1α</td>
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<td>ICAM-1</td>
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<td>CCACTGTTGAGGGAGAAATGCT</td>
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<tr>
<td>IL-1β</td>
<td>CCTACTGCTGCGCGTGGAGA</td>
<td>CCAGAACAGCGACTGTAAC</td>
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<tr>
<td>MCP-1</td>
<td>CTCCTCAGCAAGTTGACTT</td>
<td>ACGGCAGCTTGGGAGTACAT</td>
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<td>PGC-1α</td>
<td>TTCTGCTGCTGATTTAGGA</td>
<td>GAGGACAGACTCAGGTAAC</td>
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<td>SOD1</td>
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<tr>
<td>SOD2</td>
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<td>TGCGTCTCAGCCACCTCTTA</td>
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<tr>
<td>TFAM</td>
<td>GAATGGTGCCGGCTGCTAGA</td>
<td>CAGATAGAGGGCTGAGGCA</td>
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<tr>
<td>TGF-β</td>
<td>CTGGCTGACCCAGCAGTACG</td>
<td>AAGCCGCTATGGGTTCCTCTT</td>
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α-SMA, α-smooth muscle actin; Col-1α, collagen type 1 subunit α; HIF-1α, hypoxia-inducible factor 1α; MCP-1, monocyte chemoattractant protein 1; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; TFAM, transcription factor A mitochondrial; TGF-β, transforming growth factor-β.

Table 2. Animal and metabolic characteristics

<table>
<thead>
<tr>
<th></th>
<th>LETO Control</th>
<th>LETO L-NAME</th>
<th>OLETF Control</th>
<th>OLETF L-NAME</th>
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<tbody>
<tr>
<td>Serum NOX, μM</td>
<td>0.33 ± 0.05a</td>
<td>0.19 ± 0.04bc</td>
<td>0.25 ± 0.04abc</td>
<td>0.13 ± 0.02b</td>
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<tr>
<td>Body weight at euthanization, g</td>
<td>419.2 ± 9.8a</td>
<td>405.3 ± 6.2a</td>
<td>588.4 ± 12.6b</td>
<td>557.4 ± 13.6b</td>
</tr>
<tr>
<td>Food consumption during treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute, g/wk</td>
<td>159.4 ± 10.9a</td>
<td>145.8 ± 9.2b</td>
<td>235.3 ± 9.6b</td>
<td>186.0 ± 12.2c</td>
</tr>
<tr>
<td>Relative, g/wk⁻¹·g body wt⁻¹</td>
<td>0.38 ± 0.03a</td>
<td>0.36 ± 0.02bc</td>
<td>0.40 ± 0.02b</td>
<td>0.33 ± 0.02b</td>
</tr>
<tr>
<td>Weight gain during treatment, g</td>
<td>18.8 ± 4.0a</td>
<td>5.8 ± 3.2b</td>
<td>34.7 ± 4.0b</td>
<td>-7.3 ± 8.5b</td>
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<tr>
<td>DEXA body fat, %</td>
<td>13.5 ± 0.7a</td>
<td>14.0 ± 0.5a</td>
<td>27.2 ± 0.8a</td>
<td>24.6 ± 0.9a</td>
</tr>
<tr>
<td>Epididymal + retroperitoneal fat pad mass, g</td>
<td>6.8 ± 0.3a</td>
<td>7.4 ± 0.5b</td>
<td>17.6 ± 2.0b</td>
<td>17.1 ± 2.2b</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>186.0 ± 13.7a</td>
<td>178.1 ± 5.7b</td>
<td>309.8 ± 23.5b</td>
<td>312.1 ± 19.7b</td>
</tr>
<tr>
<td>Plasma insulin, mg/ml</td>
<td>8.1 ± 1.2a</td>
<td>10.1 ± 1.6b</td>
<td>32.0 ± 3.8a</td>
<td>22.4 ± 3.1b</td>
</tr>
<tr>
<td>Plasma FFA, mM</td>
<td>0.31 ± 0.03a</td>
<td>0.32 ± 0.04a</td>
<td>0.61 ± 0.04a</td>
<td>0.61 ± 0.03b</td>
</tr>
<tr>
<td>Plasma TAG, mg/dl</td>
<td>40.1 ± 2.4a</td>
<td>142.8 ± 10.9b</td>
<td>162.5 ± 9.0b</td>
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different among groups (Fig. 2, B and C). However, S1177 phosphorylation of eNOS (p-eNOS) at the constitutively active site was significantly elevated in L-NAME-treated OLETF rats compared with the other groups. This increase in p-eNOS was related to an increase in activated T172-phosphorylated AMPK (Fig. 2E), but not S473-phosphorylated Akt (Fig. 2F), known kinases of eNOS at S1177 (5, 9, 17). In addition, NO is known to modulate hepatic vascular resistance (33); thus NOS inhibition may affect hepatic perfusion and/or oxygenation. To address this possibility, we assessed hepatic hypoxia-inducible
factor 1α mRNA content and observed no differences among groups (Fig. 2f).

In an effort to understand the underlying mechanism by which l-NAME treatment caused excess hepatic TAG accumulation, we assessed the functional capacity of isolated hepatic mitochondria. Remarkably, l-NAME treatment caused a differential effect on mitochondrial respiration in OLETF vs. LETO rats (Fig. 3a). In isolated hepatic mitochondria, l-NAME treatment resulted in a significant increase in mitochondrial state 3 (glutamate + malate + ADP + succinate) respiratory rate in LETO rats but a reduction in OLETF rats (P < 0.05; Fig. 3a). A similar nonsignificant trend was observed in the other mitochondrial respiration states. In addition, isolated hepatic mitochondria from l-NAME-treated LETO rats demonstrated significantly increased complete palmitate oxidation compared with the LETO control group (Fig. 3b), and no treatment effect was observed in OLETF rats. There were no differences between groups in mitochondrial content as assessed in whole liver homogenate via OXPHOS cocktail (Fig. 3c), cytochrome c, and COX4 subunit 1 (data not shown). Furthermore, the activities of rate-limiting enzymes of the tricarboxylic acid cycle (citrate synthase) and β-oxidation (β-hydroxyacyl-CoA dehydrogenase) measured in mitochondrial isolates were not different among groups (data not shown). l-NAME treatment in LETO rats caused a ~50% reduction in peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) mRNA relative to the LETO control group. PGC-1α mRNA in the OLETF group was reduced 75% relative to the LETO control group, and, interestingly, no further reduction was observed in l-NAME-treated OLETF rats (Fig. 3d). In contrast, transcription factor A mitochondrial (TFAM) expression was significantly elevated in the OLETF control group relative to the other groups (Fig. 3e). l-NAME treatment caused a significant increase in hepatic SOD activity in LETO rats, whereas there was a trend (P = 0.13) for l-NAME to reduce hepatic SOD activity in OLETF rats (Fig. 4a). In addition, there was a trend for SOD1 and SOD2 mRNA to differ among groups (P = 0.09 and 0.15, respectively), with levels in l-NAME-treated OLETF rats tending to be reduced compared with those in the OLETF control group (Fig. 4, b and c).
In an attempt to identify other processes that could explain the increase in liver TAGs in L-NAME-treated OLETF rats, we assessed markers of hepatic de novo lipogenesis (ACC and FAS), lipid export (MTTP), fatty acid import (CD36), and skeletal muscle insulin sensitivity (Akt activation). Hepatic MTTP content did not differ among groups (Fig. 5B). CD36 protein content was significantly reduced in L-NAME-treated OLETF rats relative to the OLETF control group (Fig. 5C).

Total ACC and FAS protein was elevated in OLETF relative to LETO rats, with no effect of L-NAME treatment (Fig. 5, D–F). Phosphorylated (inactive) ACC relative to total ACC was reduced in the OLETF control group relative to the LETO control group, and this reduction was partially prevented with L-NAME treatment in OLETF rats (Fig. 5E). Finally, L-NAME treatment significantly increased the S473 phosphorylation of Akt in red gastrocnemius (RG) skeletal muscle from LETO rats, and a similar trend was observed in OLETF rats (Fig. 5G).

DISCUSSION

The progression in NAFLD severity to NASH is strongly linked to liver-related and all-cause mortality; thus efforts to elucidate the mechanisms underlying this progression are critical. We previously reported an association between reduced hepatic eNOS activation and NASH development in 40-wk-old diabetic OLETF rats that was not apparent at younger ages.
This prompted us to test the hypothesis that chronic inhibition of NOS in the OLETF rat earlier in the disease progression would induce a NASH-like phenotype. Consistent with this hypothesis, 4 wk of L-NAME treatment in insulin-resistant OLETF rats increased hepatic TAG accumulation, impaired hepatic mitochondrial respiration, and induced a shift toward M1 macrophage polarity. Remarkably, we report a different effect in lean LETO rats, where L-NAME treatment reduced expression of markers of anti-inflammatory M2 macrophage polarity, increased hepatic mitochondrial respiration, and increased hepatic SOD activity. These results demonstrate a novel role for hepatic NO in the adaptation to obesity in the liver. Efforts to exploit hepatic NO production and signaling may have important clinical implications in the treatment of NAFLD.

Our group (32) and others (16, 20, 24) have demonstrated that caloric restriction in OLETF rats rapidly and effectively improves systemic metabolic parameters and resolves hepatic steatosis. Similarly, in the current investigation, L-NAME treatment reduced food intake in OLETF rats and was associated with reduced adiposity and insulinemia (Table 1) (23). Conversely, these rats accumulated 40% more hepatic TAG content than control OLETF rats. In contrast, a previous report, 12 wk of L-NAME treatment (100 mg·kg\(^{-1}\)·day\(^{-1}\)) in mice fed a high-fat diet (80% saturated fat) caused a reduction in liver TAG content compared with controls fed a high-fat diet.
but not in various adipose tissue depots, following L-NAME administration (23), which is in contrast to our finding in the previous report. In fact, in rats fed a high-fat diet, treatment with the iNOS-specific inhibitor 1400W induced a nearly threefold reduction in liver TAG (25). In this regard, the current report is unique, as OLETF rats were studied prior to the onset of robust hepatic inflammation to gain insight into the role of constitutive, rather than inducible, NO production in NAFLD. Indeed, the vast majority of hepatic NOS activity was observed to be constitutive (Ca2+-dependent, primarily eNOS), rather than inducible, across all groups (Fig. 2F). Thus our results point to a novel protective role for hepatic constitutive NOS in NAFLD.

In addition to hepatic NOS activity, we assessed the effects of L-NAME treatment on hepatic expression of NOS isoforms. Neither eNOS nor iNOS protein content was different among groups. nNOS protein was not detected by Western blotting, suggesting that constitutive hepatic NOS activity is primarily due to eNOS. However, the phosphorylation and activation status of eNOS was enhanced in L-NAME-treated OLETF rats. This increase was associated with increased activated AMPK, a kinase of eNOS at S1177 (5), which can promote glycolytic pathway activity to maintain energy homeostasis in mitochondrial failure (1). In light of the current finding that L-NAME impaired mitochondrial function in OLETF rats (see below), these data suggest that proper hepatic NO production is essential for the hepatic response to metabolic excess.

The increase in liver TAG content in L-NAME-treated OLETF rats led us to hypothesize that NO is essential for normal hepatic lipid handling. Thus we focused on identifying mechanisms whereby NO could influence liver fat accumulation. Common indexes of hepatic de novo lipogenesis (ACC and FAS), fatty acid uptake (CD36), and TAG export (MTTP) do not explain the current data (Fig. 5). In addition, L-NAME treatment actually reduced fasting insulin (Table 2), reduced homeostatic model assessment of insulin resistance (HOMA-IR) (23), and enhanced Akt phosphorylation in skeletal muscle (Fig. 5G). Furthermore, it is unlikely that L-NAME treatment increased hepatic exposure to and uptake of FFAs due to elevated systemic lipolysis, because serum FFAs did not differ with L-NAME treatment and reduced hepatic CD36 protein expression likely indicates suppressed hepatic FFA uptake (1). Finally, we previously demonstrated in these animals an increased proinflammatory gene expression profile in the aorta, but not in various adipose tissue depots, following L-NAME administration (23), which is in contrast to our finding in the present study that L-NAME increased hepatic proinflammatory CD11c expression (Fig. 1) and suggests a novel effect of L-NAME that is somewhat unique to the liver. Instead, L-NAME treatment in OLETF rats reduced respiratory function in isolated hepatic mitochondria, which was independent of changes in mitochondrial content, β-oxidation, or tricarboxylic acid cycle function. Importantly, mitochondrial dysfunction is a key feature underlying NAFLD development and progression (30) and is present in patients with NASH (27). Thus, although the specific mechanism remains unclear, available data suggest that NOS inhibition caused a reduction of hepatic mitochondrial respiratory capacity, leading to the accumulation of intrahepatic TAG in the hyperphagic OLETF rat.

An unexpected, but intriguing, observation of the current investigation is that, in contrast to the OLETF rats, L-NAME-treated LETO rats exhibited an increase in hepatic mitochondrial respiration. Physiological NO concentrations can modify ETC flux through competitive reversible inhibition of COX (3, 14). Speculatively, we propose a model in which 1) excess nutrient availability in obesity increases driving pressure (i.e., reducing equivalents) through the ETC and 2) NO inhibition increases ETC conductance through removal of the tonic inhibitory influence of NO on COX. As a result, the combined effect of increased driving pressure and increased electron conductance (L-NAME-treated OLETF rats) is greater than that of increased driving pressure (control OLETF rats) or electron conductance (L-NAME-treated LETO rats) alone. Increased respiration without altered ATP demand will increase the mitochondrial membrane potential, which can cause superoxide generation and ETC dysfunction (12, 13). Thus one interpretation of the current findings is that NO inhibition leads to increases in mitochondrial respiration in lean normophagic LETO rats that compensated by increasing hepatic SOD activity to prevent mitochondrial reactive oxygen species (ROS)-induced damage. Alternatively, the obese hyperphagic OLETF rats were unable to adapt in the same manner, resulting in hepatic mitochondrial dysfunction, inflammation, and TAG accumulation. These differential findings suggest that the metabolically unhealthy OLETF rats were more susceptible to the negative effects of the systemic NO inhibition, but whether that was related to the environment of overnutrition or obesity or both needs more thorough examination. Future work should also include short-term (e.g., 1, 3, and 5 days) and long-term studies of NOS inhibition in OLETF and LETO rats with assessment of in vivo ROS production, mitochondrial membrane potential, and mitochondrial respiration to determine if L-NAME rats respond in the short-term similar to the LETO rats and whether longer-term NOS inhibition in LETO animals would eventually cause hepatic mitochondrial ROS-induced damage and suppression of mitochondrial function. To support this concept involving eNOS and the nutritional environment, our preliminary data show that hepatic mitochondrial respiration is higher in eNOS knockout mice fed a low-fat diet but lower in those fed a Western (high-fat, high-sucrose, and high-cholesterol) diet than in wild-type controls (R. S. Rector, unpublished observations).

One cellular response to counter mitochondrial damage/dysfunction is an increase in mitochondrial turnover. NO stimulates mitochondrial biogenesis via a cGMP/PGC-1α-dependent mechanism (19). Indeed, we observed a 50% reduction in PGC-1α mRNA in L-NAME-treated LETO rats compared with controls but no effect of L-NAME on PGC-1α mRNA in OLETF rats. Interestingly, TFAM mRNA was elevated in control, but not L-NAME-treated, OLETF rats. Given that indexes of mitochondrial content were not different between groups, these data suggest that increased mitochondrial turnover may be an adaptive mechanism to increase the amount of new, healthy mitochondria entering the mitochondrial pool. Thus it appears that L-NAME-treated OLETF rats have both an increased propensity for mitochondrial dysfunction and a relative inability to replace damaged mitochondria. This possibility warrants future study.

Another differential effect of L-NAME in lean vs. obese rats was the impact on hepatic macrophage polarization. L-NAME increases in L-NAME treatment on hepatic expression of NOS isoforms.
treatment in OLETF rats significantly increased the mRNA expression of a key M1 marker, CD11c, which is strongly associated with insulin resistance and systemic inflammation (26). In LETO rats, l-NAME treatment caused a significant reduction of M2 markers (CD163 and CD206) but had no effect on M1 markers. Along these lines, the increase in MCP-1 expression in l-NAME-treated LETO rats may represent a compensatory response to replenish the M2 pool. These findings encourage further evaluation of hepatic macrophage polarization as it relates to NAFLD and NO bioavailability.

A limitation of the current study is that l-NAME treatment is well known to cause hypertension in rats (18). The lack of acetylcholine-induced vasodilatation in isolated aortic rings from the current cohort of l-NAME-treated rats (23) would be consistent with the notion that these animals were hypertensive. Although our data are consistent with functions attributed to NO [e.g., reduced PGC-1α expression (19) and modulation of mitochondrial respiration (3, 4)], we cannot rule out the possibility that hypertension influenced the current results. However, previous work in pigs indicates that l-NAME increases hepatic resistance and blood pressure, yielding no net change in hepatic perfusion (15), a concept that is supported by decreases hepatic resistance and blood pressure, yielding no net function of the liver to adapt to an environment of overnutrition and obesity. Chronic inhibition of NOS in OLETF rats caused deleterious hepatic outcomes, including a marked increase in steatois, reduced mitochondrial respiratory capacity, and evidence of increased M1 macrophage polarity. In addition, the current data reveal a novel obesity–NO interaction in the liver, as NOS inhibition caused different effects in lean LETO rats. This work may have important therapeutic implications, as it suggests that currently available NO-based therapies may exert pleiotropic effects in the livers of obese patients.

In conclusion, the results of the current study reveal an important and novel role for constitutive hepatic NOS-mediated NO production in the ability of the liver to adapt to an environment of overnutrition and obesity. Chronic inhibition of NOS in OLETF rats caused deleterious hepatic outcomes, including a marked increase in steatois, reduced mitochondrial respiratory capacity, and evidence of increased M1 macrophage polarity. In addition, the current data reveal a novel obesity–NO interaction in the liver, as NOS inhibition caused different effects in lean LETO rats. This work may have important therapeutic implications, as it suggests that currently available NO-based therapies may exert pleiotropic effects in the livers of obese patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.D.S., J.P., N.T.J., M.H.L., and R.S.R. developed the concept and designed the research; R.D.S., J.P., N.T.J., M.H.L., and R.S.R. performed the experiments; R.D.S. and R.S.R. analyzed the data; R.D.S., J.P., N.T.J., M.H.L., and R.S.R. interpreted the results of the experiments; R.D.S. prepared the figures; R.D.S. and R.S.R. drafted the manuscript; R.D.S., J.P., N.T.J., M.H.L., and R.S.R. edited and revised the manuscript; R.D.S., J.P., N.T.J., M.H.L., and R.S.R. approved the final version of the manuscript.

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