

Published in final edited form as:

Neurogastroenterol Motil. 2011 August ; 23(8): 773–e335. doi:10.1111/j.1365-2982.2011.01695.x.

Impairment of Nitroergic System and Delayed Gastric Emptying in Low Density Lipoprotein Receptor (LDLR) Deficient Female Mice

Pandu R. Gangula^{1,2}, Vijayakumar Chinnathambi¹, Ashley B. Hale², Sutapa Mukhopadhyay¹, Keith M. Channon², and Kalpana Ravella¹

¹ Department of Physiology, Meharry Medical College, Nashville, TN, USA

² Center for Women's Health Research, Meharry Medical College, Nashville, TN, USA

² Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Abstract

Background—In the current study, we have investigated whether low density lipoprotein receptor knockout mice (LDLR-KO), moderate oxidative stress model and cholesteremia burden display gastroparesis and if so whether nitroergic system is involved in this setting. In addition, we have investigated if sepiapterin (SEP) supplementation attenuated impaired nitroergic system and delayed gastric emptying.

Methods—Gastric emptying and nitroergic relaxation were measured in overnight fasting mice. nNOS α dimerization, anti-oxidant markers such as Nrf2, GCLM, GCLC, HO-1, catalase (CAT) and superoxide dismutase (SOD1) were measured using standard methods. Biopterin levels and intestinal transit time were measured using HPLC and dye migration assay, respectively. Wild type (WT) and LDLR-KO were supplemented with SEP.

Key Results—In LDLR null stomachs, 1) significant reduction in rate of gastric emptying, gastric pyloric and fundus nitroergic relaxation & nNOS α dimerization, 2) elevated oxidized biopterins and reduced ratio of BH₄/BH₂+B, 3) reduced Nrf2 and GCLC protein expression & no change in GCLM, HO-1, Cat, Sod1 and 4) accelerated small intestinal motility were noticed. Supplementation of SEP restored delayed gastric emptying, impaired pyloric and fundus nitroergic relaxation with restoration of nNOS dimerization and nNOS expression.

Conclusions and Inferences—This novel data suggests that hyperlipidemia and/or suppression of selective antioxidants may be a potential cause of developing gastroparesis in diabetic patients.

Keywords

Gastroparesis; LDLR-KO; nitroergic relaxation; nNOS dimerization; Nrf2; sepiapterin

Correspondence and reprint requests: Pandu R. Gangula, PhD, Associate Professor, Department of Physiology, Meharry Medical College, 1005 Dr. D.B. Todd Jr. Blvd, Nashville, TN-37208, pgangula@mmc.edu; Ph: 615-327-6511.

DISCLOSURES

There are no conflict of interest to disclose for the authors except Pandu R. Gangula (the University of Texas Medical Branch, Galveston, TX has filed a patent application in his name). Pandu R Gangula is involved in the design of experiment, and the interpretation of results as well as writing the manuscript. Kalpana Ravella, Vijayakumar Chinnathambi and Sutapa Mukhopadhyay were involved in conducting some of the experiments as well as helping in writing the manuscript. Ashley Hale and Keith Channon were involved in conducting biopterin analysis using HPLC system.

INTRODUCTION

Low-density lipoprotein (LDL) is a type of lipoprotein that transports cholesterol and triglycerides from liver to peripheral tissues¹. The LDL receptor (LDLR) is a cell-surface receptor that mediates the endocytosis of cholesterol-rich intermediate density lipoproteins (IDL) and LDL from plasma.² Deficiency of LDLR display elevated plasma cholesterol level, IDL and LDL without a significant change in high density lipoproteins and triglyceride levels.^{3, 4} which increases lipid storage in the body system¹⁻³ that may eventually predisposes to oxidative stress and diabetes.⁵

Gastroparesis is a clinical condition which is associated with abnormal gastric motility and is affecting predominantly young women.⁶ Gastric motility is regulated in large part by the enteric nervous system (ENS) mainly, excitatory (acetylcholine) and inhibitory system [nitric oxide (NO) and vasoactive intestinal peptide].⁷ NO produced by neuronal nitric oxide synthase (nNOS) and its importance in gastric function has been established by the findings of pyloric hypertrophy and gastric dilation in *nNOS*^{-/-} mice.^{8, 9} The catalytic activity of NOS depends on dimerization with help of (6R)-tetrahydrobiopterin (BH₄).^{10, 11} Enzymatic uncoupling of NOS or reduced NOS dimerization due to lack of BH₄ may in part account for lower NO production and increased oxidative stress factors such as superoxide.¹² We have recently reported that BH₄ synthesis is reduced in diabetic female and leading to impaired nNOS activity and delayed gastric emptying for solids which has been restored by supplementation of BH₄.^{13, 14}

Nrf2 (NF-E2-related factor 2) is a transcription factor that protects the cells from oxidative stress by activating the phase II antioxidant enzymes (Glutamate-cysteine ligase catalytic, (GCLC) and modifier (GCLM) subunits and HO1) through ARE (antioxidative responsive element).¹⁵ Recent work suggests that the Nrf2 is critical for protecting GI tract against disease by regulating a multifaceted cellular antioxidant defense.^{16, 17} Studies also have shown that NO donors activates the transcription up-regulation of phase II enzymes, through Nrf2 via ARE in vascular endothelial cells.¹⁸

Recently it has been reported that VLDLR expression in skeletal muscle and heart is reduced in streptozotocin (STZ) induced diabetic rats while there was no change in LDL-receptor expression, as assessed in liver.¹⁹ However, mice with STZ induced diabetes and LDL receptor defects often develop severe hyperlipidemia.^{20, 21} It has also been reported that the severe elevation of serum cholesterol and TG concentrations in STZ-induced diabetic rats.¹⁹ And it is a known fact that exaggerated postprandial hypertriglyceridemia is common in diabetic patients and this may also contribute for the development of gastroparesis.⁵

Treatment of diabetic vascular endothelial cells with sepiapterin (SEP) (the BH₄ precursor in the salvage pathway) significantly improves NO synthesis and cholinergic vascular relaxation in diabetic mice.²² Recently, we have reported that, sepiapterin reverses the changes in gastric nNOS dimerization and function in diabetic gastroparesis²³. However, there have been no studies on the effects of SEP in LDLR-KO mice associated with gastroparesis. In the present study, we investigated whether deficiency of LDLR is involved in the pathogenesis of gastroparesis through nNOS uncoupling due to altered biopterin levels associated with antioxidant(s) suppression and if so supplementation of SEP attenuated impaired gastric nitrenergic relaxation and delayed gastric emptying.

MATERIALS AND METHODS

Experimental mice

Adult female C57BL LDLR-KO homozygous (-/-) (12 wk old) and their wild type (WT) (+/+) were procured from Jackson laboratories (Sacramento, CA) and maintained in the institutional animal care facility under controlled temperature, humidity and light-dark cycle (12:12 h), with free access to rodent chow and water. The protocols for using these mice were approved by the Institutional Animal Care and Use Committees at the Meharry Medical College, Nashville, Tennessee, in accordance with the recommendations of National Institutes of Health, Guide for the Care and Use of Laboratory Animals. WT and LDLR-KO animals were provided with SEP tablets (20 mg/Kg body wt/day) for 11 days. Sepiapterin tablets were given daily to the animal(s) those were placed individually in a separate cage (1 gram tablet made in the diet with chocolate flavor). These tablets were given to each animal every day in the morning hours (between 9.00 AM to 10 AM) prior to providing their regular rat chow. On the last day of SEP (Schircks Laboratories, Switzerland) supplementation, gastric emptying and gastric fundus nitrergic relaxation studies were performed. Animals were sacrificed by cervical dislocation to collect gastric fundus and antrum muscular tissue for future analysis. Tissue samples collected from animals were snap frozen in liquid nitrogen and stored at -80°C until analyzed.

Solid gastric emptying studies

Solid gastric emptying studies were performed as reported earlier.^{13, 14} Briefly, after fasting for overnight known amount of normal diet was fed to the animals for 3 h. At the end of 3 h, the remaining food was collected and calculated the amount of food intake. Then animals were fasted again for 2 h without food and water. At the end of fasting, animals were sacrificed and gastric tissues were isolated and measured the weight of the whole stomach. Then food contents were removed by opening the stomach and measured the empty stomach weight. The rate of gastric emptying was calculated according to the following equation: gastric emptying (% in 2h) = $(1 - \text{gastric content food intake}^{-1}) \times 100$. Following this protocol, we have noticed that 4 out of 5 animals displayed delayed gastric emptying.

Measurement of glucose

Drop of blood was collected from the tail vein fasted (2 h, 12 h) LDLR-KO and WT mice without anesthesia for determination of plasma glucose levels using Alphatrack™ Blood monitoring pack.

Measurement of stool frequency and weight

Small intestinal motility was measured according to the method of Li *et al.*²⁴ Briefly, each mice was placed in a clear plastic cage for 1 h. Stools were collected immediately after expulsion and placed in sealed tubes. The total stools were weighed to provide a wet weight, then dried overnight at 65°C and weighed again to provide a dry weight.

Small intestine transit

Following a 12 h fast, each animal was administered 0.1 mL of methylene blue labeled 10 % glucose by oral gavage. After sacrifice (0–30 min after gavage) the animals, stomachs were clamped with a string tied both at the lower esophageal sphincter and the pylorus to prevent dye leakage. The small intestine was removed and intestine transit was assessed by measuring the distance the dye front had traveled from the pylorus.^{25, 26}

Measurement of biopterin levels

Determination of Biopterins in Gastric Antrum Muscular Tissue—Biopterin levels were determined in antrum homogenates by HPLC followed by electrochemical and fluorescent detection, as described previously.²⁷ Briefly, samples were homogenized in phosphate-buffered saline (50 mM L⁻¹), pH 7.4, containing dithioerythritol (1 mM L⁻¹) and EDTA (100 μM L⁻¹). Following centrifugation (15 min at 13,000 rpm and 4 °C), the samples were transferred to new, cooled micro tubes and precipitated with cold phosphoric acid (1 M L⁻¹), trichloroacetic acid (2 M L⁻¹), and dithioerythritol (1 mM L⁻¹). The samples were vigorously mixed and then centrifuged for 15 min at 13,000 rpm and 4 °C. The samples were injected onto an isocratic HPLC system and quantified using sequential electrochemical (Coulchem III, ESA Inc.) and fluorescence (Jasco) detection. HPLC separation was performed using a 250 mm, ACE C-18 column (Hichrom) and mobile phase comprising of sodium acetate (50 mM L⁻¹), citric acid (5 mM L⁻¹), EDTA (48 μM L⁻¹), and dithioerythritol (160 μM L⁻¹) (pH 5.2) (all ultrapure electrochemical HPLC grade) at a flow rate of 1.3 mL/min. Background currents of +500 μA and -50 μA were used for the detection of BH4 on electrochemical cells E1 and E2, respectively. 7,8-BH2 and biopterin were measured using a Jasco FP2020 fluorescence detector. Quantification of BH4, BH2, and biopterin was done by comparison with authentic external standards and normalized to sample protein content.

Organ Bath Studies

Electric field stimulation (EFS)-induced NANC relaxation was studied in circular gastric fundus and pylorus strips as reported earlier.^{13, 14} Strips were tied with silk thread at both ends and were mounted in 10 ml water-jacketed organ baths containing Krebs buffer at 37°C and continuously (Danish Myo Technology, USA). Tension for each muscle strip bubbled with 95% O₂ - 5% CO₂ was monitored with an isometric force transducer and analyzed by a data acquisition system (Power Lab, ADI, USA). A passive tension equal to 2 g was applied on each strip in the 1h equilibration period through an incremental increase (0.5 g, four times, at 15 min interval). Strips were exposed to atropine, phentolamine and propranolol (10 μM each) in bath solution for 1 h to block cholinergic and adrenergic responses. 5-hydroxytryptamine (100 μM) pre-contracted strips were exposed to EFS (90V, 2Hz, 1-ms pulse for duration of 1 min) to elicit NANC relaxation by low frequency (2 Hz) The NO dependence of nitrergic relaxations was confirmed by preincubation with NG-nitro-L-arginine methyl ester (L-NAME, 100 μM; 30 min). At the end of each experiment, the muscle strip was blotted dry with filter paper and weighed. Comparisons between groups were performed by measuring the area under the curve (AUC mg tissue⁻¹) of the EFS-induced relaxation (AUC_R) for 1 min and the baseline for 1 min (AUC_B) according to the formula (AUC_R - AUC_B) weight of tissue (mg)⁻¹ = AUC mg of tissue⁻¹.

Western blot analysis

nNOS α , GTPCH-1, DHFR, Nrf2, GCLC, GCLM and HO-1 protein was quantified in gastric antrum homogenates from the 2 groups using standard western blot analysis, as described in our previous study.^{13, 14} Proteins were measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA) and 30 μg protein was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The membrane was immunoblotted with polyclonal nNOS α (Zymed Laboratories Inc., CA), Nrf2, GTPCH-1, DHFR, GCLC, and GCLM primary antibodies (Santacruz Biotechnology, CA) and anti-rabbit IgG conjugated with horseradish peroxidase (Sigma Chemical, St. Louis, MO) as secondary antibody. Binding of antibodies to the blots was detected with enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Piscataway, NJ) following manufacturer's instructions. Stripped blots were re-probed with γ -tubulin specific polyclonal antibodies (Sigma Chemical, St. Louis, MO) to

enable normalization of signals between samples. Band intensities were analyzed using Bio-Rad Gel Doc (Bio-Rad, Hercules, CA).

nNOS α dimerization in mice gastric antrum

Levels of nNOS α monomer and dimer were quantified by western blotting via Low temperature (LT)-PAGE in gastric antrum homogenates as described previously.⁶ LT-SDS-PAGE was performed on ice. 30 μ g of protein in standard Laemmli buffer was incubated at 0°C for 30 min and then separated by using a 6% separating gel. All gels and buffers were pre-equilibrated to 4°C prior to electrophoresis and the buffer tank placed in an ice-bath during electrophoresis to maintain the gel temperature below 15°C. A polyclonal antibody specific to nNOS α (Zymed Laboratories) and anti-rabbit IgG conjugated with horseradish peroxidase (Sigma Chemical, St. Louis, MO) were used as the primary and secondary antibodies, respectively.

Activity of antioxidant enzymes in LDLR-KO mice

Stomach antrum was homogenized in cold 50 mM potassium phosphate, pH 7.0. The homogenate was centrifuged at 10,000 \times g and 4°C for 15 min and the supernatant was separated to measure total protein and the activities of catalase (CAT). Cat activity in was assayed by a commercially available kit from Cayman Chemical Company (Ann Arbor, MI) and the data were expressed as nmol min⁻¹ mg protein⁻¹. For total superoxide dismutase (SOD1) activity, tissue was homogenized in 20 mM HEPES buffer, pH 7.2. The homogenate was centrifuged at 1,500 \times g for 5 min at 4°C and the supernatant was separated to measure total protein and the activities of Sod1. Sod1 activity in stomach antrum was assayed by a commercially available kit from Cayman Chemical Company (Ann Arbor, MI) and the results were expressed as U mg protein⁻¹.

Statistics

Data were presented as mean \pm standard error (SE). Statistical comparisons between groups were determined by Student's *t*-test using Graph pad prism Version 5.0 (Graph pad software, San Diego, CA). A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Body weights and glucose levels in LDLR-KO mice

We first investigated the body weights and glucose levels difference among the female LDLR-KO and age-matched WT mice. As shown in Table 1, no significant difference in body weight was observed in LDLR-KO mice compared to wild type mice. Compared to WT mice, LDLR-KO mice showed slightly higher plasma glucose levels under fasted conditions (2 h and 12 h), and suggesting impaired glucose tolerance in LDLR-KO mice (Table 1).

Delayed gastric emptying in LDLR-KO mice

Fig. 1 shows the solid gastric emptying (% in 2 hours) in female LDLR-KO mice. A significant delay (58 ± 6.11) in the solid gastric emptying was observed in LDLR-KO mice compared to WT (78 ± 0.88) animals. Our results show that supplementation of sepiapterin (79.30 ± 2.97) restored delayed gastric emptying in LDLR-KO mice whereas no significant change in sepiapterin treated WT mice (75 ± 0.33).

Transient increase in small intestinal motility in LDLR-KO mice

The LDLR-KO mice didn't show any change in stool frequency in one hour after 12 h fasting than WT animals (Fig. 2A). Similarly, the stool weight also not much differ among LDLR-KO and WT mice (Fig. 2B). Whereas the intestine transit time was significantly increased in LDLR-KO mice when compared with WT mice at the time point of 5 min, interestingly it didn't show much difference at 30 min interval (Fig. 2C).

Elevated oxidized biopterin levels in LDLR-KO mice

Fig. 3A shows the levels of total biopterins (BH₄, BH₂ and B) in WT and LDLR-KO mice. No significant change was observed in BH₄ level, whereas a significant increase in BH₂ as well as B levels was observed in LDLR-KO mice compared to WT mice. The ratio of BH₄ and total biopterin was significantly reduced in LDLR-KO mice compared to WT animals. No significant change in the expression of GTPCH-1 and DHFR was observed in LDLR-KO mice (Figs. 3B & C)

Impairment of gastric fundus and pyloric nitrenergic relaxation in LDLR-KO mice

Fig. 4A shows the nitrenergic relaxation in fundus muscular tissue following EFS (2 Hz) in WT, SEP supplemented WT mice, LDLR-KO mice and sepiapterin (SEP) supplemented LDLR-KO mice. The gastric fundus nitrenergic relaxation was impaired in female LDLR-KO mice compared to WT mice (-0.28 ± 0.01 vs. -0.12 ± 0.02). Whereas, supplementation of SEP resulted in complete reversal (-0.23 ± 0.02) of impaired nitrenergic relaxation in female LDLR-KO mice, but no effect on wild animals (-0.25 ± 0.02). Fig. 4B shows the nitrenergic relaxation in pyloric muscular tissue following EFS (2 Hz) in WT, SEP supplemented WT mice, LDLR-KO mice and sepiapterin (SEP) supplemented LDLR-KO mice. The gastric pyloric nitrenergic relaxation was impaired in female LDLR-KO mice compared to WT mice (-0.02 ± 0.02 vs. -0.20 ± 0.07). Whereas, supplementation of SEP (-0.13 ± 0.07) significantly ($p < 0.05$) reversed of impaired nitrenergic relaxation in female LDLR-KO mice, but no effect on wild animals (-0.19 ± 0.07).

Decreased gastric antrum nNOS α protein expression and dimerization in LDLR-KO mice

According to Fig. 4C the protein level of nNOS α , the only functional isoform of nNOS in gastric antrum muscular tissue, was significantly ($p < 0.05$) decreased (0.28 ± 0.03 vs 0.39 ± 0.04) in LDLR-KO mice when compared to WT whereas, supplementation of SEP resulted in reversal of nNOS expression (0.38 ± 0.03) in female LDLR-KO mice. To measure whether decrease in the nNOS α was the result of altered nNOS dimer levels in LDLR-KO mice, we performed the dimerization study by LT-PAGE gel. As depicted in Fig. 4D, a significant ($p < 0.05$) decrease in the dimer/monomer ratio was seen in LDLR-KO mice compared to WT mice (0.17 ± 0.03 vs 0.48 ± 0.03) whereas, its restored in SEP supplemented LDLR-KO (0.42 ± 0.05) animals.

Reduced gastric antrum expression of Nrf2 in LDLR-KO mice

Fig. 5A demonstrates the expression of Nrf2 in LDLR-KO mice. The protein level of Nrf2 was reduced significantly in LDLR-KO mice (0.24 ± 0.04 vs. 0.62 ± 0.01) when compared to WT mice.

Reduced gastric antrum expression of GCLC in LDLR-KO mice

The protein levels of GCLC in WT and LDLR-KO mice were shown in Fig 5B. The protein level of GCLC was significantly reduced in LDLR-KO mice (0.6 ± 0.03 vs. 1.14 ± 0.06) when compared to WT, a phase II enzyme whose expression is regulated by Nrf2. However, no significant change in the protein expression of GCLM and HO-1 was observed in LDLR-KO mice (Figs 5C & 5D).

Activity of antioxidant enzymes (CAT & SOD1) in LDLR-KO female mice

SOD1 is a family of ubiquitous antioxidant enzymes that catalyze the conversion of superoxide anion radicals to hydrogen peroxide (H₂O₂) and molecular oxygen, whereas, CAT is a ubiquitous antioxidant enzyme that is involved in the conversion of H₂O₂ to molecular oxygen and water. No significant change was observed in both the activities of CAT as well as SOD1 in LDLR-KO mice compared with age matched WT animals (Fig. 5E).

DISCUSSION

Gastroparesis is a disorder of delayed gastric emptying, with the most common known cause being diabetes. Recently using streptozotocin-induced diabetic model (Type 1 diabetes in humans), we have reported that impairment in nitrenergic relaxation and reduction in nNOS dimerization (critical of enzyme activity and subsequent nitric oxide production), enzyme activity and altered bipterin levels are responsible for developing gastroparesis in female rats.^{13, 14} Hyperlipidemia as well as oxidative stress may be some of the detrimental causes for observed impaired gastric motility dysfunctions associated with diabetes and it has not been addressed previously. Oxidative stress which not only interplays with NO ability to activate sGC (soluble guanylate cyclase) but also NO bioavailability because superoxide anion quenches NO, transforming it into peroxynitrite.²⁸

This is the first comprehensive report of gastrointestinal dysmotility in LDLR-KO mice, an animal model of hyperlipidemia. The elevation of hyperlipidemia status and its associated molecules in the GI tract perhaps may leads to consistently depletion of nitrenergic function. The accelerated small intestinal motility observed in LDLR-KO mice (Fig. 2) is consistent with the loss of nitrenergic neuronal function in the GI tract.^{13, 14} However; additional studies are warranted to investigate the role of nitrenergic neurons in this setting.

Although, elevated hyperlipidemia is one of the detrimental factor for diabetes induced complications; to date there are no literature available if this impairs bipterin and nitrenergic system and thus leading to gastroparesis. In the current study, we have first investigated whether LDLR-KO mice display gastroparesis and if so nitrenergic system is involved in gastric emptying. The present study demonstrates that gastric emptying is delayed in female LDLR-KO mice compared to WT mice and it's restored by supplementation with SEP (Fig. 1). In addition, our data demonstrates that impaired nitrenergic relaxation observed in LDLR-KO mice was restored by SEP supplementation (Fig. 4A & B). Since the predominant form of nNOS in the enteric nerves of the stomach is nNOS α , we next investigated if the expression and dimerization of nNOS is altered in LDLR-KO mice.¹³ As shown in Fig 4, both nNOS α expression (Fig. 4C) and nNOS α dimerization (Fig. 4D) was impaired in LDLR-KO mice; supplementation of SEP restored this. The above data for the first time suggest that, hyperlipidemia causes impairment in nitrenergic mediated gastric motility and leads to gastroparesis; SEP treatment may be beneficial to regulate the gastric motility by modulating the lipid metabolism. Further studies are warranted to address this in detail.

BH₄, an essential co-factor for NOS is intracellularly produced from GTP via GTP-cyclohydrolase I (GTPCH-1) or, alternatively, from SEP via salvage pathway and acts as a redox switch in the oxygenase domain of NOS.²⁹ Reduced levels of BH₄ impair the production of NO, and leads to increased superoxide radical production, due to nNOS uncoupling. The superoxide radical then reacts with NO resulting into the production of peroxynitrite. This further reduces biological availability of NO.³⁰ BH₄ deficiency has been associated with diabetic complications including gastroparesis.¹⁴ This information provides a rational basis for the use of supplemental sepiatperin (SEP; a precursor for BH₄ biosynthesis via salvage pathway) in diabetic conditions. In this study, we demonstrate that

supplementation of SEP normalizes both delayed gastric emptying (Fig. 1) and impaired nitregeric relaxation in female LDLR-KO mice (Fig. 4). This is in good agreement with our previous study, where we showed that supplementation of BH₄ or SEP restored the delayed gastric emptying associated with diabetes in female rats.^{13, 14, 23} Though BH₄ levels have not been altered in LDLR-KO mice, the finding that significant increase in the oxidized biopterin levels followed by a decrease BH₄:BH₂+B level (Fig. 3A) may ultimately reduce the BH₄ bioavailability. Several studies support the notion that a decrease in the ratio of BH₄:BH₂ without changing in absolute BH₄ levels are associated with increased vascular diseases.^{22, 31} Elevated oxidized biopterins (BH₂, B) not only reduces the bioavailability of NOS cofactor BH₄, but also competes with BH₄ to bind to NOS domain that may eventually down regulates the dimerization of the enzyme and function.¹⁴ In addition, GTPCH1 and DHFR gene knock out studies demonstrated that either major reductions in de novo BH₄ biosynthesis or an altered BH₄:BH₂ ratio is sufficient to induce eNOS uncoupling.³² The data reported in the current studies also demonstrate that a reduction in the ratio of BH₄ vs BH₂+B may be responsible for decreased nNOS dimerization, nitregeric relaxation and thus leading to delayed gastric emptying.

To investigate the factors involved in reduced BH₄ bioavailability we checked the expression of GTPCH (GTP cyclohydrolase, which converts GTP to 7,8-dihydroneopterintriphosphate) and DHFR (dihydro folate reductase which converts 7,8-dihydrobiopterin to BH₄) in both control, LDLR-KO mice tissues. As shown in Figs. 3B & C, we did not observe any change in the expression of GTPCH, DHFR in LDLR-KO mice. Apart from these observations, our data indicate that in LDLR-KO mice gastric oxidized biopterin levels (BH₂, B) but not BH₄ have been elevated (Fig. 3A). Collectively, these results suggests that elevated hyperlipidemia status leads to increased gastric oxidized biopterins and nNOS uncoupling which results in impaired nitregeric relaxation and thus gastroparesis.

We also noticed a significant decrease in the expression levels of both Nrf2 as well as phase II enzymes, GCLC which is essential for the synthesis of glutathione (GSH) in LDLR-KO mice (Figs. 5A & B). Nrf2 is a transcriptional factor that protects the cells from oxidative stress by activating the antioxidant enzymes including GSH synthesis enzymes (GCLC, GCLM) which is critical for the protection of neuronal cells. Genes such as heme oxygenase-1 (HO-1), SOD1, CAT, and Gpx1 are representative of canonical antioxidant genes regulated by Nrf2. Recent work suggests that the Nrf2 is critical for protecting GI tract against disease by regulating a cellular antioxidant defense.^{16, 17} The above findings may suggest that reduced levels of phase II enzymes elevate oxidative stress and thus impairs both nitregeric system and gastric motility functions. We can also conclude that chronic depletion of LDLR may be one of the detrimental factors in the pathogenesis of gastroparesis due to increased hyperlipidemia. This is in good agreement with previous results which states that Nrf2 inhibits lipid accumulation and oxidative stress in mouse liver after feeding a high fat diet, probably by interfering with lipogenic and cholesterologenic pathways.³³ Recent studies have shown that HO-1 reverses gastroparesis in Type 1 diabetic mice by protecting against oxidative stress.^{34, 35} However, data from LDLR-KO stomachs show that no change in the expression of antioxidant enzymes HO-1 (Fig. 5D) and activity of CAT & SOD1 (Fig. 5E) may explain that this is not a severe oxidative stress model; instead probably it is a moderate stress model. Previous studies have demonstrated that high fat fed LDLR-KO displays hyperglycemia, severe hypertriglyceridemia and elevated oxidative stress.³ The molecular mechanisms responsible for decreased nNOS dimerization and/or how LDL influences gastric motility functions are not currently understood. Recent studies have demonstrated that more than 50% of patients with idiopathic gastroparesis are obese or overweight.³⁶ These studies further report that women had more gastroparesis symptoms than in men; however, the etiological connection is completely unknown.³⁶ In

addition, loss of ICC and/or reduced nNOS containing neurons that are noticed in diabetic rodents may also account for the impairment of stomach motility and delay in gastric emptying in LDLR null mice.^{37–38} Additional experiments are warranted to address in detail with regard to mechanisms of nNOS regulation as well as the potential role for NO on gastric smooth muscle function in both normal and high fat fed LDLR-KO mice. The results obtain by using LDLR-KO animal model may provide an opportunity to understand the molecular mechanisms that are involved in idiopathic gastroparesis.

Acknowledgments

We thank to Freeman Michael, PhD Konjeti, Rajasekhar, PhD (Vanderbilt University Medical Center) and Farrugia Gianrico, M.D (Mayo Clinic) for scientific discussions. Finally, we thank Shanthi Srinivasan, M.D and Anitha Mallappa (Emory University) for providing us the intestinal transit protocol as well as guidance on completing the transit experiments.

Financial Support for the original research was provided by the NIH-NIDDK R21DK076704 (PG), P60DK020593 pilot project funds (PG) and from RCMI G12RR03032 (PG), as start-up funds at Meharry Medical College, Nashville, TN, USA.

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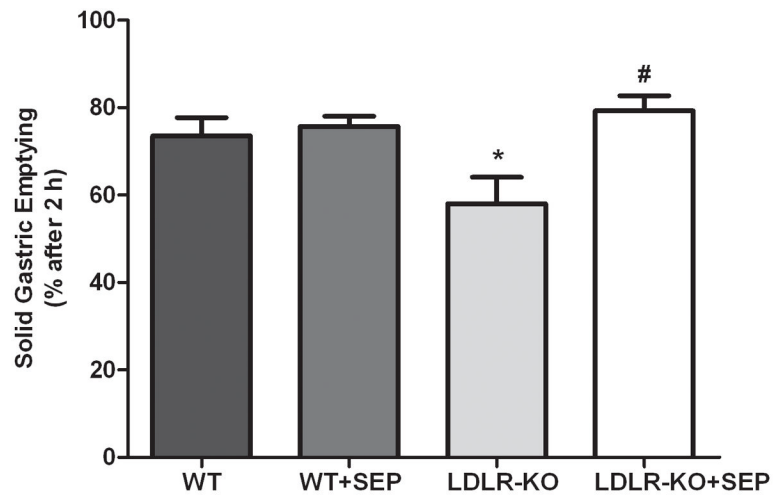


Fig.1. Solid gastric emptying in female wild type (WT), LDLR-KO, sepiapterin (SEP) supplemented WT mice and sepiapterin (SEP) supplemented LDLR-KO mice. The values are mean \pm SE for four animals in each group. Statistical significance was determined by Student t-test. Values are mean \pm SE (n = 4). *p<0.05 compared with WT animals and #p<0.05 compared with LDLR-KO animals.

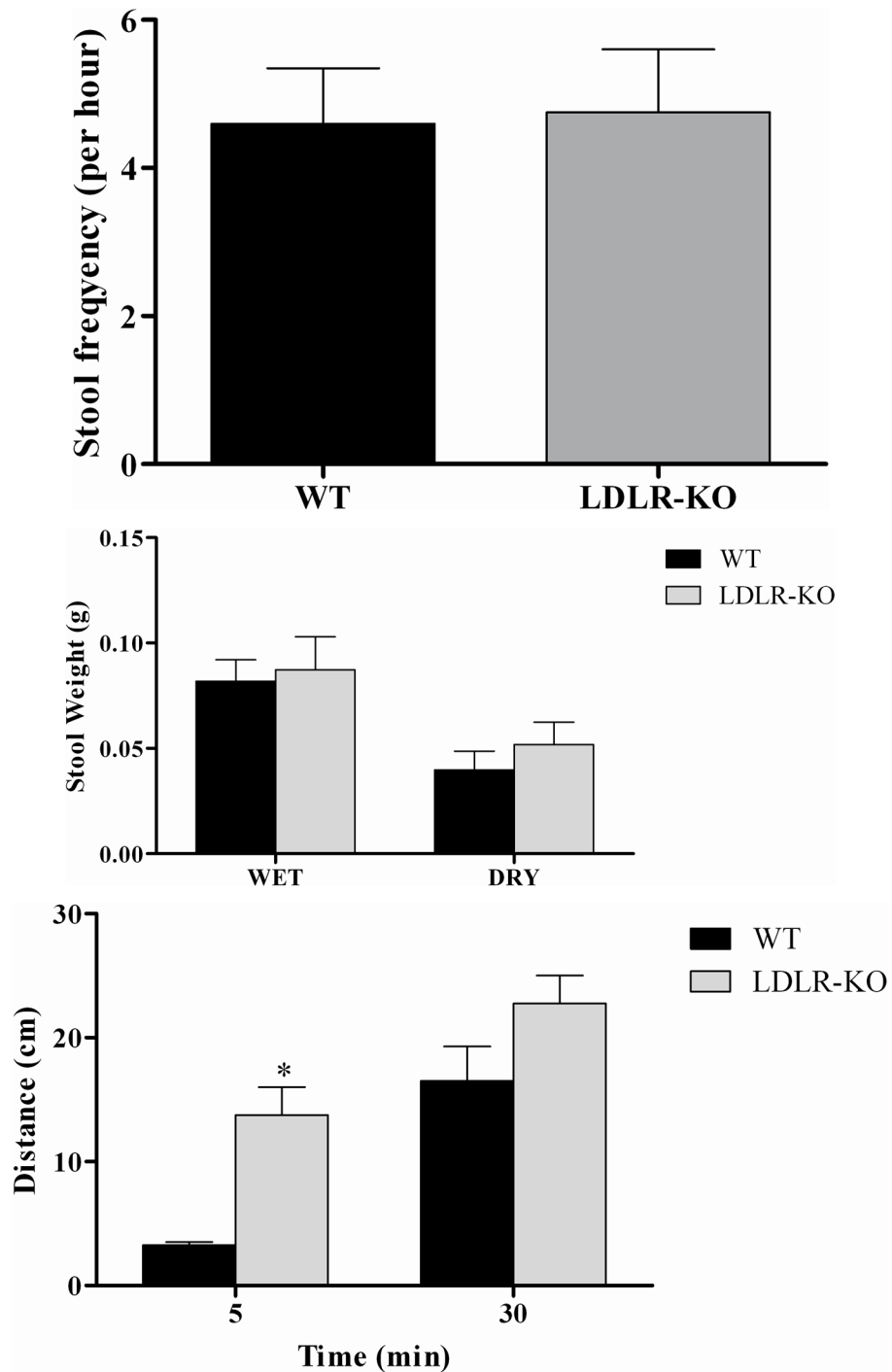
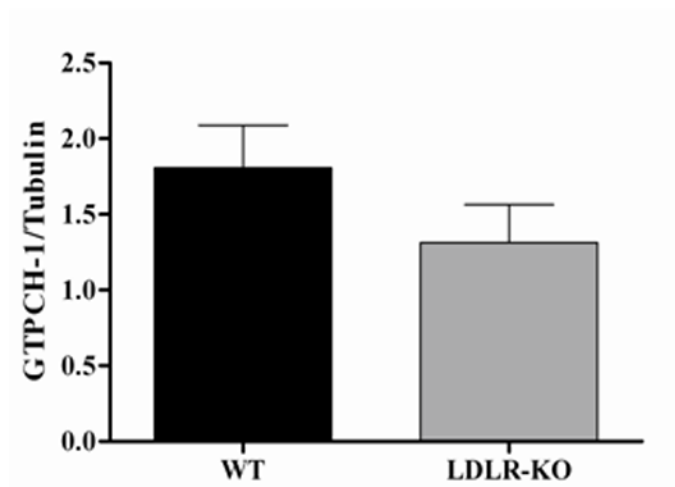
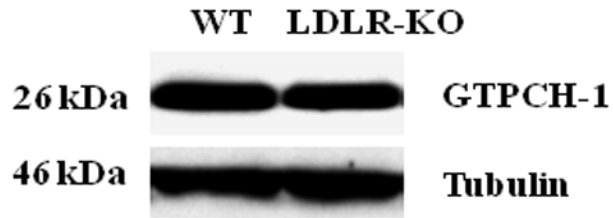
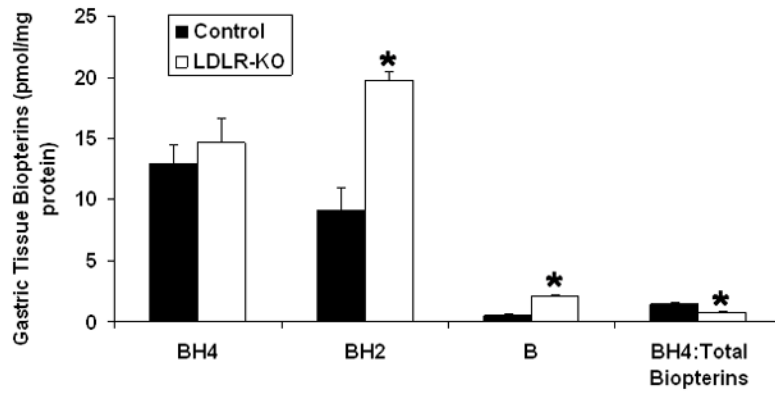


Fig. 2. (A). Stool frequency was monitored in LDLR-KO mice and wild type (WT) mice for one hour period. Since the small intestinal functions to remove water, this confirms the utility of 1 h stool frequency as a measure of colon transit time. (B) The stool weight was collected during one hour period and it expressed weight in gm in LDLR-KO and WT mice. (C) The intestine transit time was monitored by measurement of dye migration in the colon.

Statistical significance was determined by Student t-test. Values are mean \pm SE (n = 5). *p < 0.05 compared with WT animals.



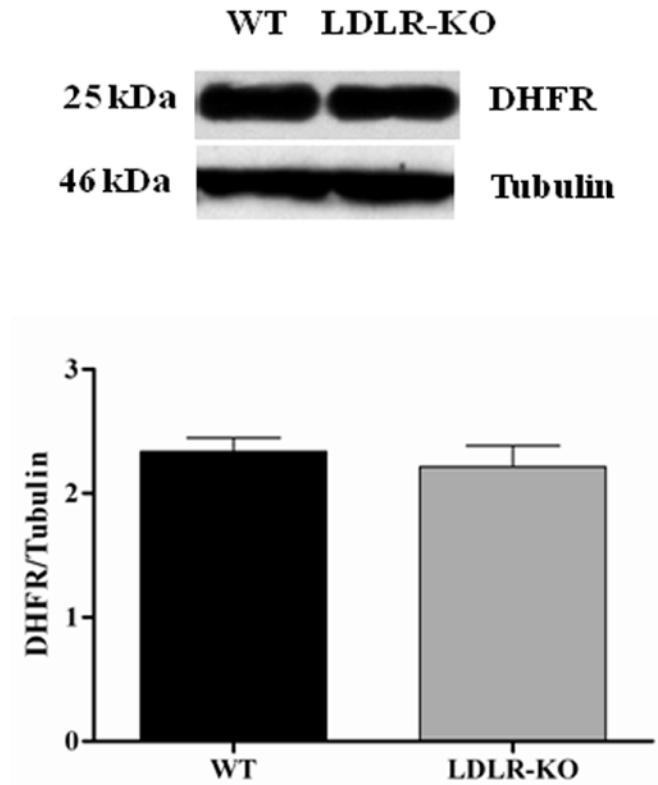
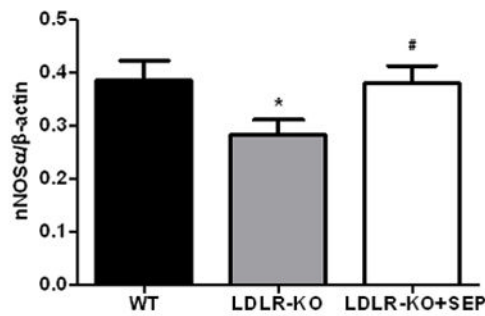
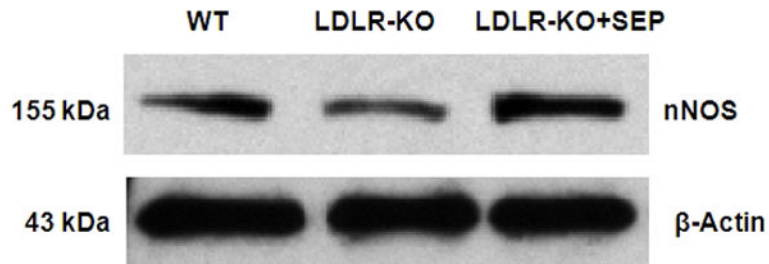
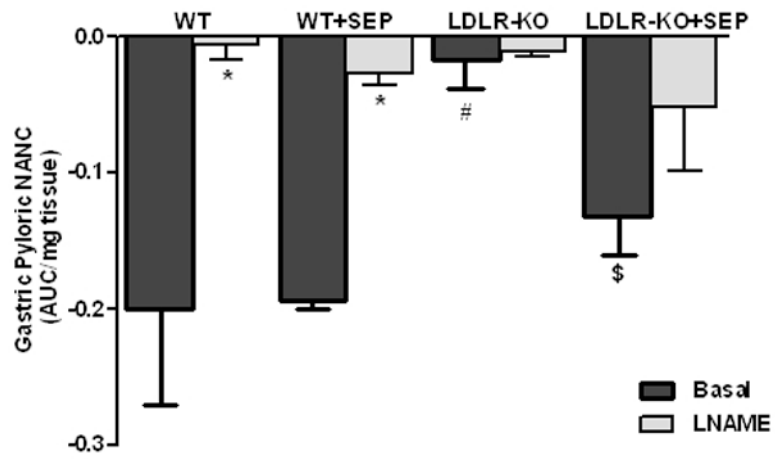
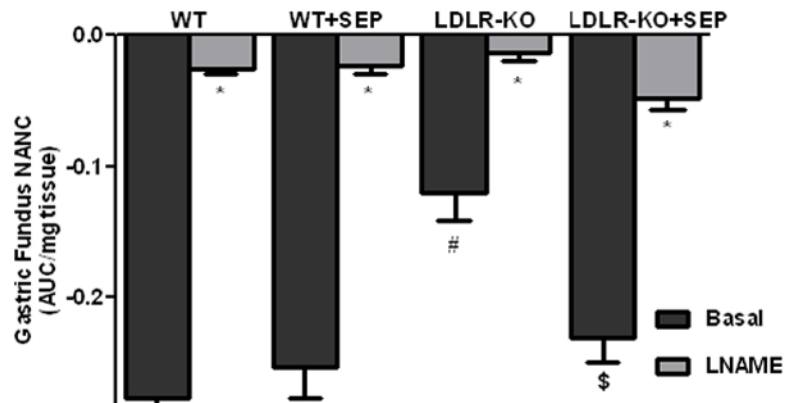


Fig. 3. (A) Levels of total biopterins in LDLR-KO female mice gastric tissue. Biopterin levels were measured in wild type (WT) and LDLR-KO female mice gastric antrum tissue using HPLC. (B) Representative immunoblot and densitometric analysis data for protein expression of BH₄ synthesizing enzymes GTPCH-1 and (C) DHFR. Statistical significance was determined by Student t-test. Values are mean ± SE (n = 4). *p < 0.05 compared with WT animals.



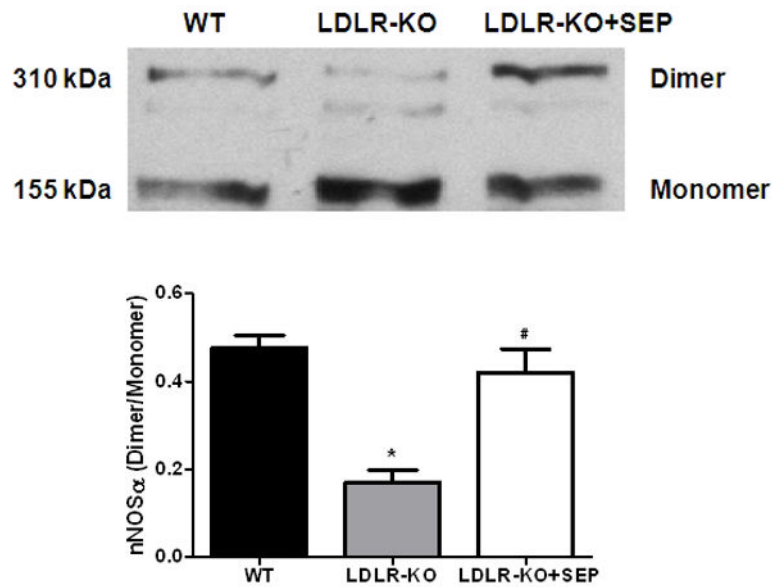
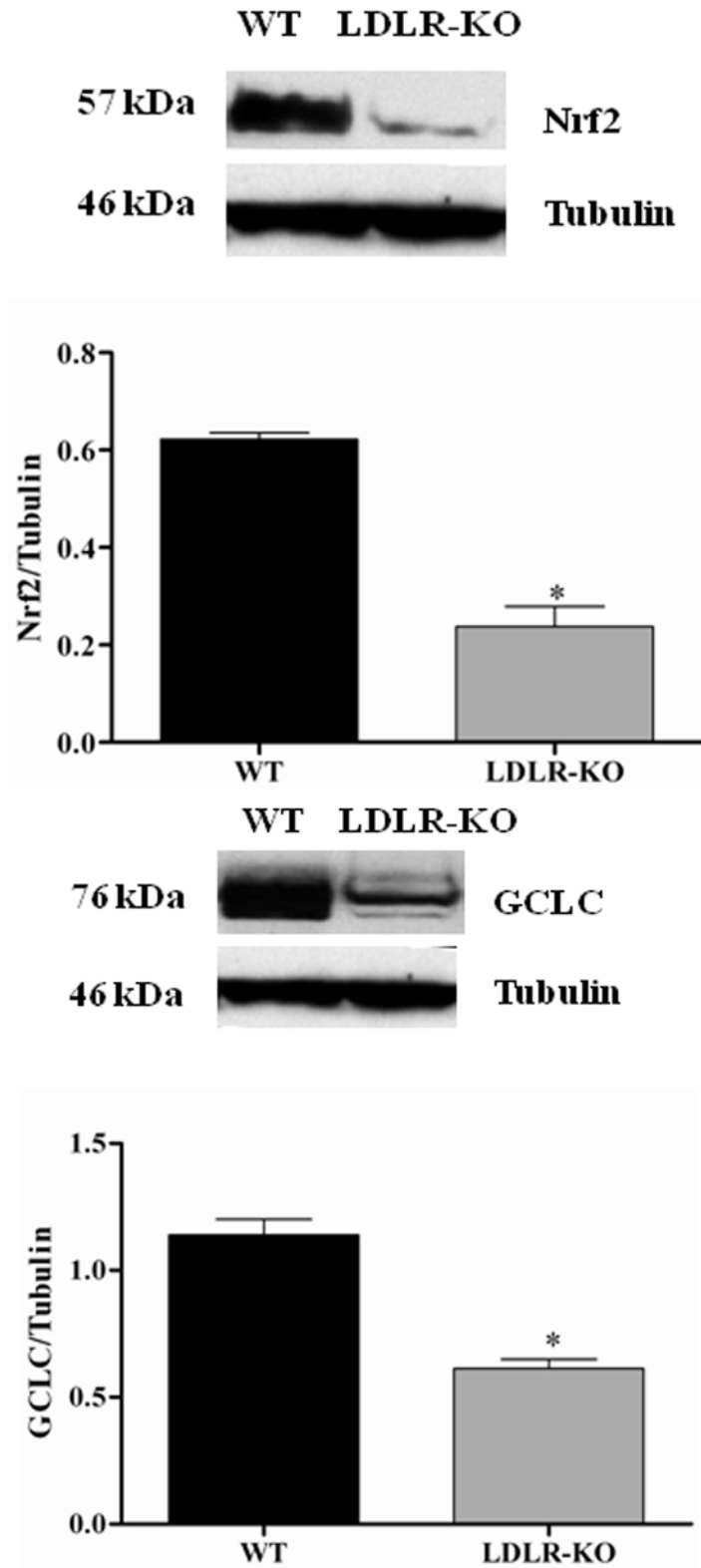
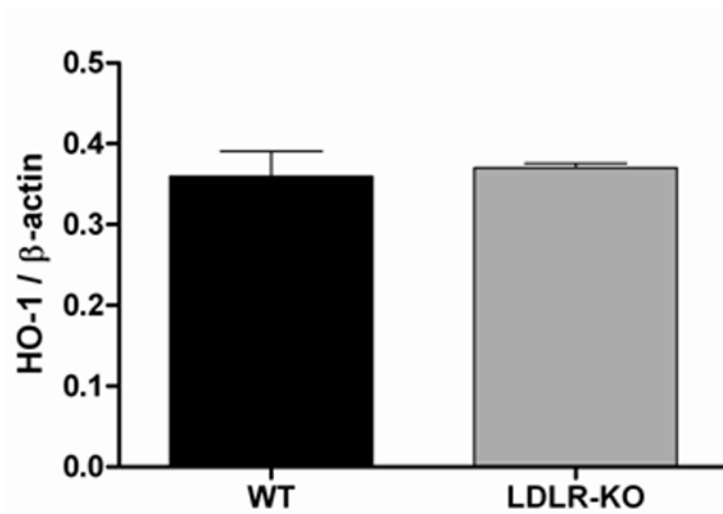
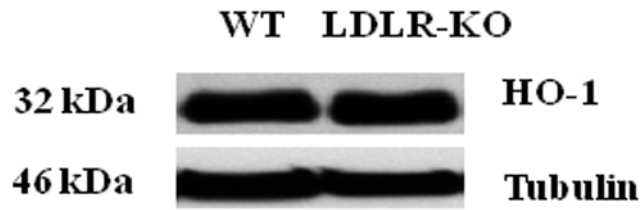
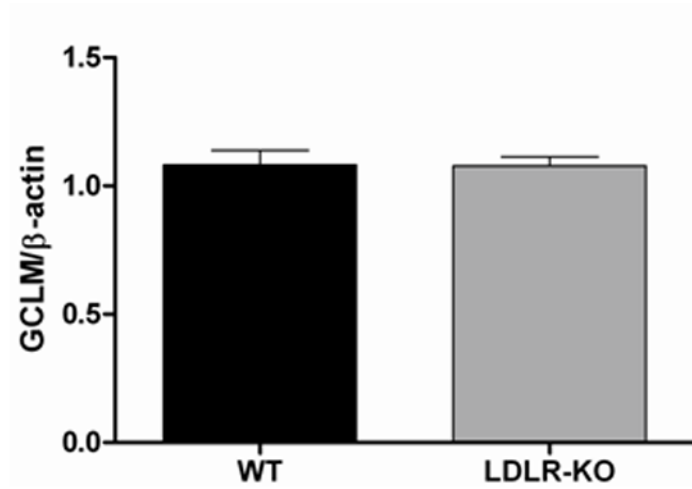
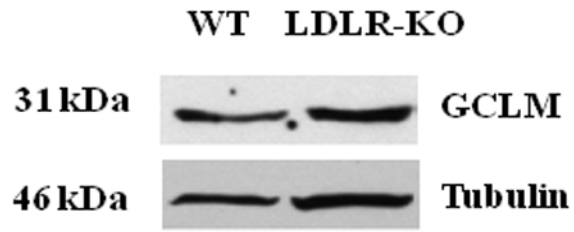


Fig. 4. (A) Impairment of nitroergic relaxation in female wild type (WT), SEP supplemented WT mice, LDLR-KO and sepiapterin (SEP) supplemented LDLR-KO mice gastric fundus muscular tissues. (B) Impairment of nitroergic relaxation in female wild type (WT), SEP supplemented WT mice, LDLR-KO and sepiapterin (SEP) supplemented LDLR-KO mice gastric pylorus muscular tissues. The nitric oxide (NO) dependence of the NANC relaxations in all groups was confirmed by preincubation (30 min) with the NO inhibitor nitro-L-arginine methyl ester (L-NAME; 100 μ M). The values are mean \pm SE of four samples in each group. Statistical significance was determined by Student t-test. Values are mean \pm SE (n = 4), * p < 0.05 compared with the response in the absence of L-NAME, # p < 0.05 compared with WT animals and \$ p < 0.05 compared with LDLR-KO animals. nNOS α protein expression and dimerization in LDLR-KO female mice gastric antrum tissues (C) Representative immunoblot and densitometric analysis data for nNOS α protein expression. (D) Representative immunoblot and densitometric analysis data for nNOS α protein dimerization. Statistical significance was determined by Student t-test. Values are mean \pm SE (n = 4), *p < 0.05 compared with WT animals and # p < 0.05 compared with LDLR-KO animals.





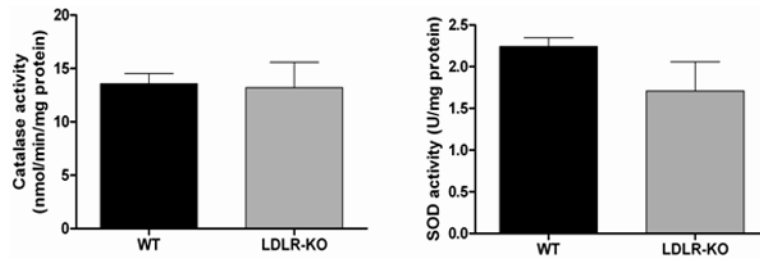


Fig. 5. Nrf2 and GCLC protein expression in LDLR-KO female mice gastric antrum tissues. (A) Representative immunoblot and densitometric analysis data for Nrf2 protein expression. (B-D) Representative immunoblot and densitometric analysis data for GCLC, GCLM and HO-1 protein expression. The values are mean \pm SE of three to four samples in each group. (E) CAT activity, and SOD1 activity in LDLR-KO female mice gastric antrum tissues. Values are mean \pm SE (n = 4). Statistical significance was determined by student t-test. *p < 0.05 compared with wild type (WT) animals.

Table 1

Blood glucose level and body weight in the wild type and LDLR-KO female mice

	WT	KO
Body Weight, g	18.5 ± 1.0	21.0 ± 0.7
Blood Glucose, mg/dl		
2 h	187.2 ± 11.1	225.5 ± 5.0*
12 h	97.4 ± 3.7	125.0 ± 11.9*

Wild type (WT); Knock out (KO). Results are expressed as mean ± SEM (N = 5).

* p < 0.05 compared to WT.