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Impairment of Gastric Nitrergic and NRF2 System in Apolipoprotein E Knockout Mice

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Abstract

Background and Aim—Gastric motility dysfunction is most commonly seen in diabetic and idiopathic gastroparesis patients. Recently we reported that impaired nitrergic relaxation and a reduced NO (nitric oxide) bioavailability were responsible for gastric motility dysfunction in diabetic female rats. One of the main factors involved in the inactivation of the nitrergic system is oxidative stress commonly seen in diabetic patients. Hyperlipidemia may also be one of the detrimental causes for impaired gastric motility associated with diabetes. In the current study, we investigated whether apolipoprotein E knockout mice (ApoE-KO), an oxidative stress animal model with a hyperlipidemia burden, also displays an impaired nitrergic system. To test this, nitrergic relaxation (AUC/mg tissue) was measured at 2 Hz through electric field stimulation using gastric pyloric strips prepared from C57BL WT or ApoE-KO female mice. Protein expression was determined by Western blots.

Results—Nitrergic relaxation was reduced in gastric strips from ApoE-KO versus WT mice. Protein levels of nNOS (neuronal nitric oxide synthase), GCH-1 (GTP cyclohydrolase 1), Nrf2 (nuclear factor E-2 related factor 2) and GCS_c (glutamate-cysteine ligase catalytic) were also reduced in ApoE-KO compared to controls, with no significant change in GCS_m (glutamate-cysteine ligase modifier) and HO-1 (heme oxygenase 1). The activities of DHFR (dihydrofolate reductase) and antioxidant enzymes were also reduced in ApoE-KO mice.

Conclusions—This novel study is the first to reveal that a deficiency in ApoE impairs gastric motility functions, and that hyperlipidemia and the suppression of selective antioxidants may be an underlying mechanism for this pathological change.

Keywords

Apolipoprotein E; Nitric oxide; Gastroparesis; Nitrergic relaxation; nNOS dimerization; Nrf2; Phase II enzymes; BH₄

Introduction

Apolipoprotein E (ApoE) is a class of apolipoprotein found among the chylomicron and intermediate-density lipoproteins that bind to a specific receptor on liver and peripheral cells. It is essential for the normal catabolism of triglyceride-rich lipoprotein constituents [1]. ApoE transports lipoproteins, fat-soluble vitamins, and cholesterol into the lymph system and then into the blood. It is synthesized principally in the liver, but has also been found in other tissues such as the brain, kidneys, and spleen. ApoE lipoproteins are ligands for interaction with low density lipoprotein receptors in tissues [2]. Defects in ApoE result in type III hyperlipoproteinemia (HLP III), in which increased plasma cholesterol and triglycerides are the result of impaired clearance of VLDL and LDL remnants leading to increased lipid storage in the body. This may eventually increase oxidative stress and lead to diabetes [3–7]. Lack of ApoE also leads to decreased antioxidant and NO levels [8, 9].

Gastroparesis is a clinical condition which is associated with abnormal gastric motility and predominantly affects young women [10–16]. We have recently reported that BH₄ availability is reduced in diabetic females and leads to delayed gastric emptying which has been restored by supplementation of BH₄ [12, 13, 17]. Elevated oxidative stress is one of the detrimental factors for diabetes induced complications. Apart from BH₄, Nrf2 (nuclear factor erythroid 2-related factor) is another vital element which is an anti-oxidant element and is involved in defense mechanisms. Nrf2 is a transcription factor that protects the cells from oxidative stress by activating the phase II antioxidant enzymes [18–21].

It has been reported that exaggerated postprandial hypertriglyceridemia is common in diabetic patients [22]. Along with LDL protein ApoE also plays an important role in the efficient production and clearance of lipoproteins. ApoE acts as a ligand for the interaction of low density lipoprotein receptors in the tissues [2, 7]. So deficiency of ApoE results in the impairment of LDLR interaction with the tissues thus leading to increased plasma cholesterol and triglycerides along with oxidative stress. Previous studies have demonstrated that ApoE serves as a direct antioxidant and protects cells from oxy radical damage [5, 7, 8, 23, 24]. There have been no studies demonstrating the role of ApoE in diabetic gastroparesis. Therefore, in this present study we have used ApoE-KO mice to demonstrate whether deficiency of ApoE is involved in the pathogenesis of diabetic gastroparesis through impairment of the nitrergic system.

Materials and Methods

Experimental Mice

Adult female ApoE-KO mice (12 weeks old) were procured from Jackson Laboratories (Sacramento, CA) and maintained in the institutional animal care facility. The protocols for using these mice were approved by the Institutional Animal Care and Use Committees at the Meharry Medical College, Nashville, Tennessee. Tissue samples collected from animals were snap frozen in liquid nitrogen and stored at –80°C.

Organ Bath Studies

Electric field stimulation (EFS)-induced NANC relaxation was studied in circular gastric pyloric strips using organ bath (Radnoti Glass Technology, Monrovia, CA, USA). Pyloric strips were used as delayed emptying is increased by abnormal pyloric contractions [12]. Strips from WT and ApoE-KO mice were collected and NANC relaxation is measured through EFS induction. The NO dependence nitregeric relaxations were confirmed by preincubation with NG-nitro-L-arginine-methyl ester (L-NAME, 100 μ M). Comparisons between groups were performed by measuring the area under the curve (AUC/mg tissue).

Western Blot Analysis

nNOS α , GCH-1, DHFR, Nrf2, GCS α , GCS β and HO-1 proteins were quantified in gastric antrum homogenates from the two groups using standard western blot analysis, as described in our previous study. Proteins were measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) and separated by SDS polyacrylamide gel electrophoresis. The membrane was immunoblotted with polyclonal primary antibodies (Zymed Laboratories Inc., CA; Santacruz Biotechnology, CA) and their respective secondary antibodies.

Activity of DHFR and Antioxidant Enzymes (SOD and Catalase) in ApoE-KO Mice

Activity of the enzyme DHFR is measured using a commercially available kit (Sigma Aldrich). CAT activity was assayed by a commercially available kit from Cayman Chemical Company (Ann Arbor, MI, USA) and the data were expressed as nmol/min/mg protein). SOD1 activity in stomach antrum was assayed by a commercially available kit from Cayman Chemical Company.

Statistics

Data were presented as mean \pm standard error (SE). Statistical comparisons between groups were determined by Student's *t* test or the Tukey test after one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered statistically significant.

Results

Gastric Pyloric Nitregeric Relaxation Is Impaired in Female ApoE-KO Compared to WT Mice

Figure 1a depicts the gastric nitregeric relaxation following EFS (2 Hz) in WT and ApoE-KO mice. The elevated levels of oxidative stress and hyperlipidemia in female ApoE-KO significantly impaired gastric nitregeric relaxation compared to WT mice (0.069 ± 0.04 vs -0.2 ± 0.07 , *P* < 0.05).

Oxidative Stress and Hyperlipidemia in ApoE-KO Decreases Gastric nNOS α Protein Expression

According to Fig. 1b the protein level of nNOS α , the only functional isoform of nNOS in gastric fundus tissue, was significantly (*P* < 0.05) decreased (0.18 ± 0.02 vs 0.34 ± 0.1) in ApoE-KO mice when compared to WT. Similarly, Fig. 1c shows the reduced expression of nNOS α protein in gastric antrum in ApoE-KO mice when compared to WT (0.22 ± 0.04 vs 0.38 ± 0.02 , *P* < 0.05). Therefore, all our subsequent experiments were carried out in gastric antrum muscular tissue.

Reduced Expression of Gastric Antrum GCH-1 in ApoE-KO Mice

Figure 2a shows a significant decrease in GCH-1 protein expression in female ApoE-KO mice gastric antrum compared to WT mice (0.49 ± 0.04 vs 0.68 ± 0.04 , *P* < 0.05).

Expression of DHFR Protein in WT and ApoE-KO Female Mice Gastric Tissue

Figure 2b shows no change in DHFR expression in ApoE-KO mice compared to WT mice (0.20 ± 0.05 vs 0.21 ± 0.05).

Reduced Expression of Gastric Antrum Nrf2 Protein in ApoE-KO Mice

Figure 3a demonstrates the expression of gastric antrum Nrf2 in ApoE-KO mice. The protein level of gastric antrum Nrf2 is reduced significantly in ApoE-KO mice (0.26 ± 0.13 vs 0.32 ± 0.016 , $P < 0.05$) as a result of high oxidative stress when compared to WT mice. We next examined if a reduction observed in Nrf2 protein alters the phase II enzyme levels in ApoE-KO gastric antrum muscular tissue.

Expression of Gastric Antrum GCSs, GCSm, HO-1 Proteins in ApoE-KO Mice

The protein levels of GCSs in WT and ApoE-KO mice gastric antrum are shown in Fig. 3b. The protein level of GCSs is significantly ($P < 0.05$) reduced in ApoE-KO mice (0.63 ± 0.02 vs 0.78 ± 0.06) when compared to WT. However, Fig. 3c and d shows no change in GCSm and HO-1 protein expression in ApoE-KO mice compared to WT mice.

Activity of DHFR and Antioxidant Enzymes (CAT and SOD1) in ApoE-KO Female Mice

Table 1 depicts gastric DHFR enzyme activity which was significantly ($P < 0.05$) reduced in ApoE-KO mice when compared to WT mice (0.006 ± 0.004 vs 0.2 ± 0.004). It also shows a reduction in SOD and CAT activity in ApoE-KO mice compared to WT mice [$(6.07 \pm 0.77$ vs 2.97 ± 0.72 , $P < 0.05$); $(6.79 \pm 0.57$ vs 5.09 ± 0.48 , $P < 0.05$), respectively].

Discussion

The aim of the present study was to demonstrate whether hypercholesteremia and suppression of gastric antioxidants in ApoE-KO mice leads to impairment in nNOS mediated stomach motility function. Recently, we reported an impairment in nitric oxide mediated relaxation and reduced expression of nNOS α protein, nNOS dimer which are responsible for the development of gastroparesis in streptozotocin-induced diabetic female rats [12, 13]. We have also reported that suppression of selective antioxidants and hyperlipidemia impairs bipterin and the nitric oxide system, thus leading to gastroparesis in LDLR-KO mice model [25]. In this study we tested whether lack of ApoE protein contributes to NO mediated gastric motility dysfunction. For the first time in the GI literature, herein we demonstrated that gastric nitric oxide mediated relaxation as well as gastric nNOS alpha expression is reduced in ApoE-KO mice. We also demonstrated that the protein expression/activity of BH₄ biosynthesis enzymes of GCH-1, DHFR (enzymes involved in the production of BH₄ via de novo and salvage pathways respectively) was reduced in ApoE-KO mice. We also noticed a significant decrease in the expression levels of phase II enzymes as well as antioxidant enzyme activity in ApoE-KO mice stomach muscular tissue. Therefore we suggest that elevated levels of oxidative stress as well as hyperlipidemia observed in diabetic and/or overweight (independent of diabetes) female patients may lead to nitric oxide mediated gastric motility dysfunction and thus gastroparesis.

This study is the first comprehensive report of gastric dysmotility in ApoE-KO mice, an animal model of hypercholesteremia. Previous studies have demonstrated that ApoE is a direct antioxidant and protects cells from oxy radical damage [23]. A decreased antioxidant level in ApoE-KO mice appears to correlate with decreased recovery after injury [7]. It has also been reported that there is an increased NO production from human monocyte macrophages in the presence of ApoE [8]. Further, in ApoE-KO mice, vascular relaxation is impaired, vascular superoxide production is increased and NO synthesis is reduced [5]. Loss of NO bioavailability leads to the development of atherosclerosis, thus increasing the risk of

cardiovascular disease [24]. However, there have been no studies demonstrating the role of ApoE in diabetic gastroparesis. In the present study, we have first investigated whether apolipoprotein knockout mice (ApoE-KO; displays oxidative stress and high blood cholesterol) display impairment in the gastric nitrergic system. The present study demonstrates that nitrergic relaxation is impaired in ApoE-KO mice along with a reduction in nNOS alpha expression (Fig. 1a, b).

Tetrahydrobiopterin (BH₄), an essential co-factor for NOS, is intracellularly produced from GTP via GTP-cyclohydrolase I or, alternatively, from sepiapterin (SP) via the salvage pathway and acts as a redox switch in the oxygenase domain of NOS [24]. Reduced levels of BH₄ impair the production of NO and lead to increased superoxide radical production [5]. BH₄ deficiency has been associated with diabetic complications including gastroparesis [13]. To investigate the factors involved in reduced BH₄ bioavailability we investigate changes in the protein expression of GTPCH (GTP cyclohydrolase) and DHFR (dihydro folate reductase) in both WT and ApoE-KO mice stomach muscular tissues. As shown in Fig. 2, GCH-1 but not DHFR protein levels were reduced in ApoE-KO stomach muscular tissues. Next, we tested the DHFR enzyme activity using an assay kit. As shown in Table 1, the activity of DHFR enzyme is significantly reduced in ApoE-KO mouse gastric muscular tissue. Collectively, these results suggest that hyperlipidemia leads to decreased availability of BH₄ and nNOS α uncoupling which results in impaired nitrergic relaxation and thus gastroparesis.

We next investigated if antioxidant elements like Nrf2 and phase II enzymes are altered in female ApoE-KO mice compared to wild type. Recent work suggests that the Nrf2 (nuclear factor erythroid 2-related factor) is critical for protecting the GI tract against disease by regulating a multifaceted cellular antioxidant defense [19]. Nrf2 is a transcriptional factor that protects the cells from oxidative stress and regulates the expression of phase II genes involved in regulating levels of reactive oxygen species [20]. Our data shows that the expression of Nrf2 and GCS α but not HO1 and GCS β enzymes are significantly reduced in ApoE-KO mice (Fig. 3). This suggests that reduced levels of selective phase II enzymes elevate oxidative stress and thus impair both the nitrergic system and gastric motility functions. Superoxide dismutase 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. Our data shows a significant reduction in SOD and catalase in ApoE-KO mice gastric tissue (Table 1).

Previous studies have reported that lack of ApoE contributes to eNOS dysfunction and thus reduced NO synthesis which leads to impaired vascular relaxation and thus leads to the development of atherosclerosis. In ApoE-KO mice, a hypercholesteromic model the superoxide derived from NADPH oxidase reacts with NO to form peroxynitrite which induces oxidative degradation of tetrahydrobiopterin. This leads to eNOS uncoupling and increased production of superoxide thus leading to oxidative stress [8, 9]. It has been well established that lack of ApoE contributes to vascular eNOS dysfunction. Further studies are warranted to investigate the mechanistic role of ApoE in nitrergic mediated gastric motility functions. Nevertheless, this novel study demonstrates that lack of ApoE protein contributes to NO mediated gastric dysmotility.

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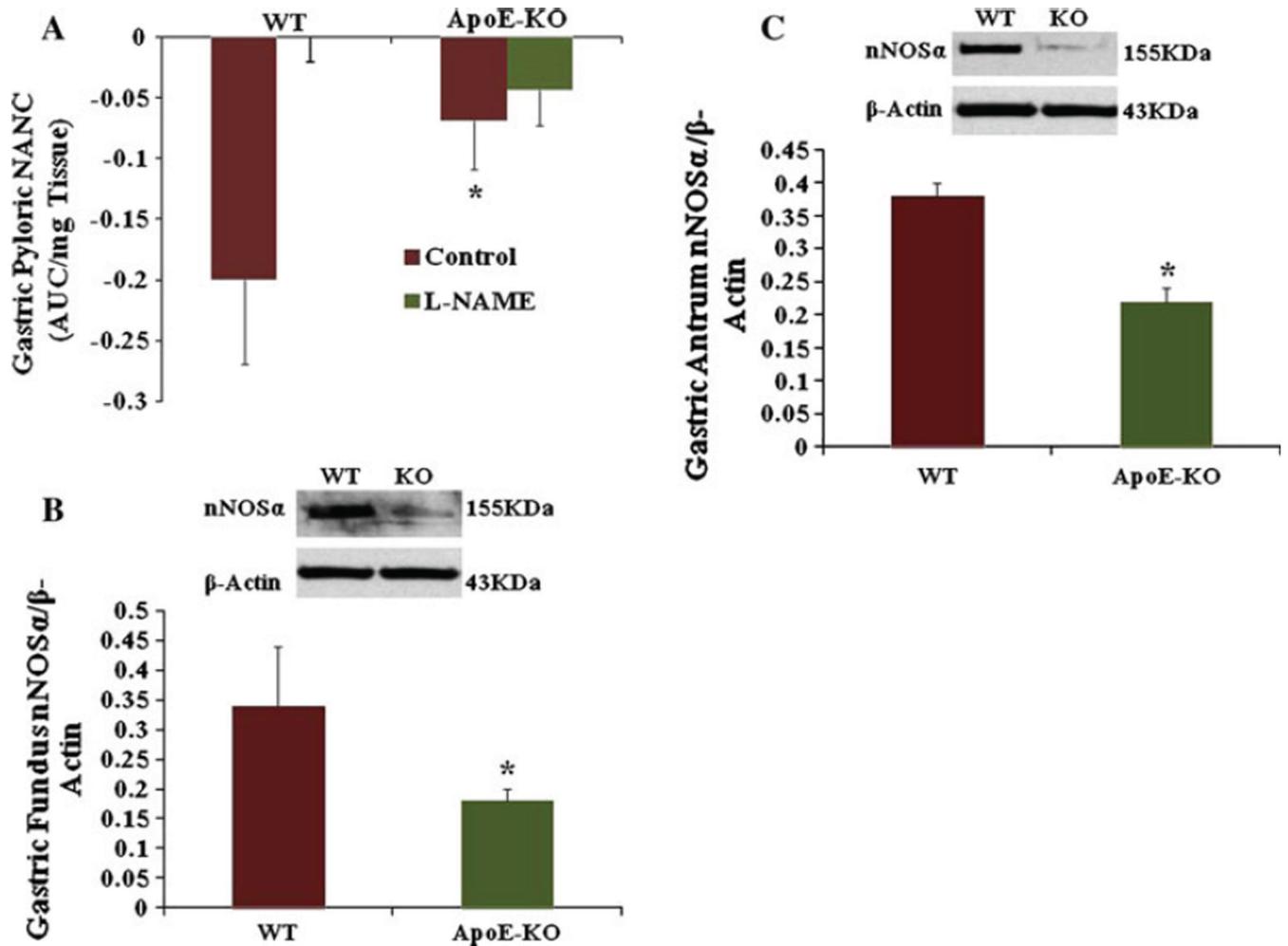


Fig. 1.

a Nitrgic relaxation in WT and apolipoprotein E knockout mice (ApoE-KO) gastric muscular pyloric tissues in vivo. Nitrgic relaxation was measured in WT and ApoE-KO pyloric gastric tissue. Nitrgic relaxation is reduced in ApoE-KO mice when compared to WT mice. **b** Expression of nNOS α in WT and ApoE-KO female mice gastric muscular tissue. Representative immunoblot and densitometric analysis data for nNOS α protein in female mice gastric fundus muscular tissue. There was a significant decrease in nNOS α in female ApoE-KO mice compared to WT mice. **c** Representative immunoblot and densitometric analysis data for nNOS α protein in female mice gastric antrum muscular tissue. A significant decrease was seen in nNOS α in female ApoE-KO mice compared to WT mice. Values are mean \pm SE ($N=4$). Statistical significance was determined by Tukey test after one-way ANOVA. * $P < 0.05$ compared with control group

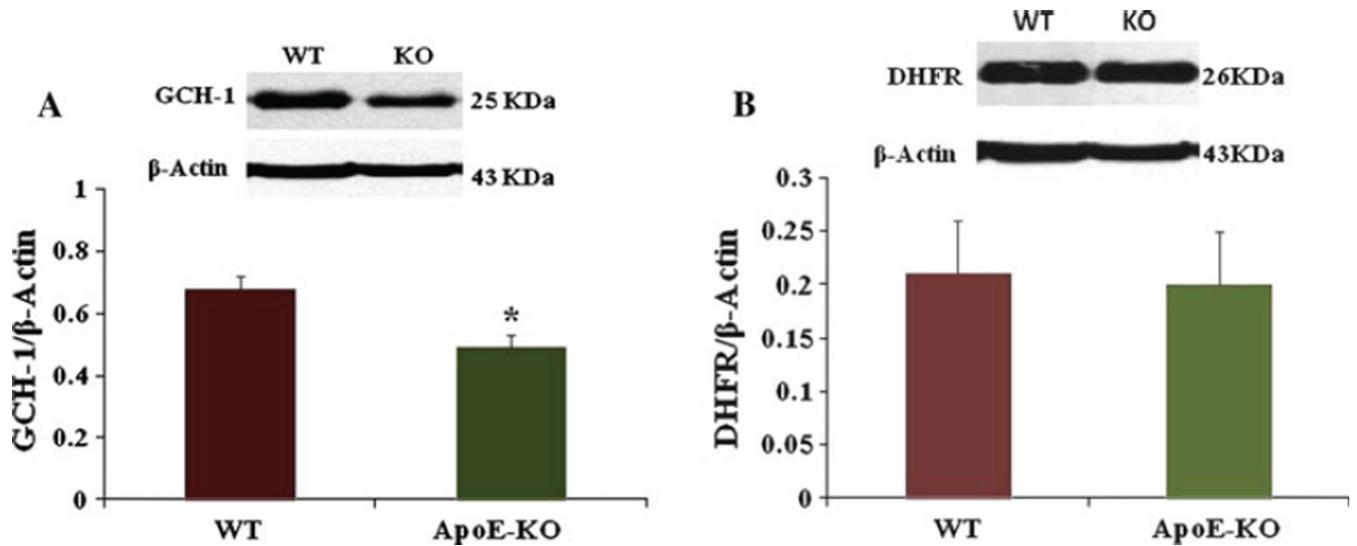


Fig. 2.

Expression of GCH-1, dihydrofolate reductase (DHFR) in WT and apolipoprotein E knockout (ApoE-KO) female mice gastric antrum muscular tissue. **a** Representative immunoblot and densitometric analysis data for GCH-1 protein in female mice gastric antrum. A significant decrease was seen in GCH-1 in female ApoE-KO mice compared to WT mice (0.49 ± 0.04 vs 0.68 ± 0.04). Values are mean \pm SE ($N = 4$). Statistical significance was determined by Tukey test after one-way ANOVA. * $P < 0.05$ compared with control group. **b** Representative immunoblot and densitometric analysis data for DHFR protein in female mice gastric antrum. Values are mean \pm SE ($N = 4$)

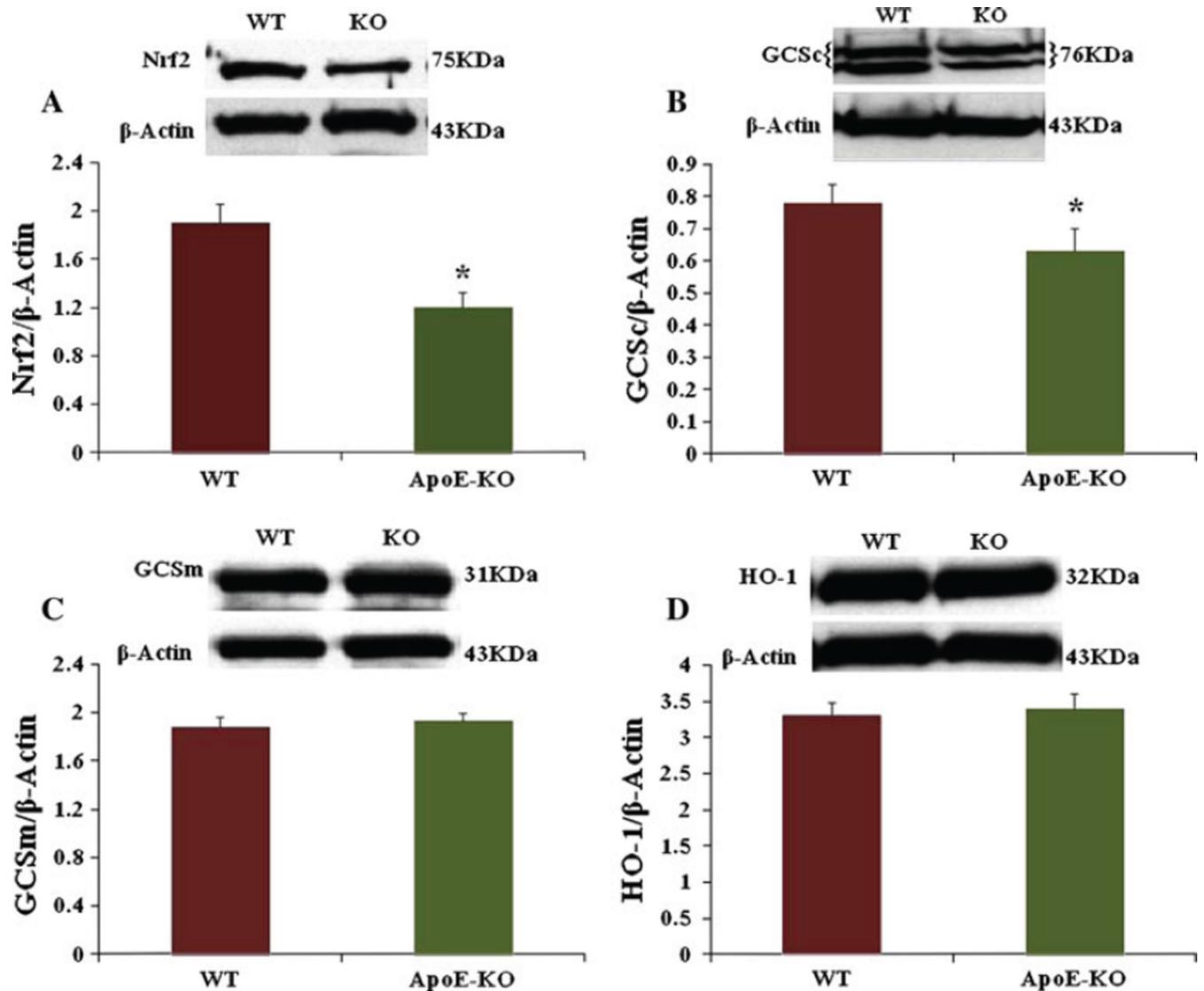


Fig. 3. Expression of Nrf2, GCSs, GCSm and HO-1 in WT and apolipoprotein E knockout (ApoE-KO) female mice gastric antrum muscular tissue. Representative immunoblot and densitometric analysis data for Nrf2 protein in female mice gastric antrum. **a** A significant decrease in Nrf2 in female ApoE-KO mice compared to WT mice. Values are mean \pm SE ($N = 4$). Statistical significance was determined by Tukey test after one-way ANOVA. $*P < 0.05$ compared with control group. **b** Representative immunoblot and densitometric analysis data for GCSs protein in female mice gastric antrum. **c** Representative immunoblot and densitometric analysis data for GCSm protein in female mice gastric antrum. **d** Representative immunoblot and densitometric analysis data for HO-1 protein in female mice gastric antrum. Values are mean \pm SE ($N = 4$)

Table 1

Activity of DHFR, antioxidant enzymes (SOD and catalase) in ApoE-KO mice gastric antrum muscular tissue. This table depicts a marked decrease of DHFR activity in ApoE-KO mice

Activity	WT	ApoE-KO
DHFR activity (micro mole/min/mg protein)	0.2 ± 0.004	0.006 ± 0.004 *
SOD activity (U/mg protein)	6.07 ± 0.77	2.97 ± 0.72 *
Catalase activity (nmol/min/mg protein)	6.79 ± 0.57	5.09 ± 0.48 *

DHFR dihydrofolate reductase, *ApoE-KO* apolipoprotein E knockout Note the significant reduction of SOD and CAT in ApoE-KO mice compared to WT mice. Values are mean ± SE ($N = 4$). Statistical significance was determined by Tukey test after one-way ANOVA.

* $P < 0.05$ compared with control group