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Activation of Nrf2 attenuates delayed gastric emptying in obesity induced diabetic (T2DM) female mice

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Abstract

Diabetic gastroparesis (GP) is a clinical syndrome characterized by delayed gastric emptying (DGE). Loss of Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) led to reduced nNOSa mediated gastric motility and DGE. The molecular signaling of cinnamaldehyde (CNM) mediated Nrf2 activation and its mechanistic role on DGE were further investigated in obese/T2D female mice. Adult female homozygous *Nfe212*$^{-/-}$ (C57BL/6J) and their wild-type (WT) littermates (*Nfe2l2*$^{+/+}$) mice were fed with high fat diet (HFD; Obese/T2D model), or normal diet (ND) with or without CNM (50mg/kg b.w; i.p). Supplementation of CNM attenuated ($p < 0.05$) DGE in WT female but not in Nrf2 KO Obese/T2D mice. CNM (1) normalized serum estradiol-17β levels, (2) induced gastric Nrf2 and phase II antioxidant enzymes through extracellular signal-regulated kinase, (ERK)/c-Jun N-terminal kinase (JNK)/p38 mitogen-activated protein kinase (MAPK), (3) reduced glucose synthase kinase 3 beta (GSK3β) and aryl hydrocarbon receptor (AhR) and this was associated with (4) increased estrogen receptor expression, BH_4 (Cofactor of nNOS) biosynthesis enzyme GCH-1 and nNOSα dimerization in WT Obese/T2 diabetic female mice. In addition, CNM restored impaired nitrergic relaxation in hyperglycemic conditions. These findings emphasize the importance of Nrf2 in maintaining nNOSα mediated GE and may have a translational relevance to treat obese/diabetic gastroparesis in women.

Keywords

Diabetic gastroparesis; Gastric emptying; Nrf2; nNOS; Estrogen receptors; GSK-3β

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Author contributions

Dr. P. R. Gangula, filed a patent application for the use of BH4 (tetrahydrobiopterin) in gastroparesis subjects through University of Texas Medical Branch at Galveston (UTMB) Texas, USA. P.G. conceived and designed research; C. S. conducted all experiments & analyzed data; P. G., and C.S. interpreted results of experiments; J. S. and M. F. edited manuscript; C. S. drafted manuscript; P.G., edited & revised manuscript; P. G. and M. F. approved final version of manuscript; J. S. assisted C.S in collecting specimens and conducting serum estradiol and NO ELISA experiments; J.S. assisted to C. S. in preparing a couple of figures for this manuscript.

Author disclosure statement

The authors declare no conflicts of interests.

1. Introduction

Gastroparesis (GP) is characterized by gastric dysmotility with delayed or accelerated gastric emptying (GE). Gastroparesis is more commonly found in patients with type-1 diabetes (insulin dependent, T1D) and obese type-2 diabetes (non-insulin dependent, Obese/T2D) [1,2]. Almost 80% of diabetic patients suffering from gastroparesis are women [3]. Although gastroparesis is a significant health problem, the molecular mechanisms responsible for gastroparesis are not well understood [4].

Increase in oxidative stress is one of the leading causes for the development of diabetes mellitus [5]. Nrf2 (NF-E2-related factor 2) is a redox-sensitive, basic leucine zipper transcriptional factor upregulates antioxidant gene expression by binding to the promoter region of the antioxidant response element (ARE) [6]. Diminished nitric oxide (NO) and enhanced oxidative stress due to lack of tetrahydrobiopterin (BH_4, a cofactor for nNOS) play a central role in several pathophysiologic pathways [7]. Deletion of nNOS resulted in delaying gastric emptying in the mice [8,9]. We have reported that loss of Nrf2 (*Nfe2/2*$^{-/-}$ female mice) resulted in decreased levels of BH_4, inhibited nNOS mediated gastric nitrergic relaxation and reduced nitrite levels which led to delayed gastric emptying [10]. These reports support the notion that loss of Nrf2 expression in diabetes impaired gastric antioxidant gene expression, nNOS function which deregulates NO synthesis, thereby contributing to the development of gastroparesis [3,10]. Earlier, we have provided evidence that depletion of estradiol-17β (E_2) impaired gastric Nrf2 expression, nitrergic relaxation and delayed gastric emptying suggesting that Nrf2/nNOS mediated gastric motility is depended on sex hormones [11]. In addition, we have reported that gastric Nrf2/BH_4/nNOS expression and nNOS function is altered in the onset of diabetes and other rodent models [12–15]. However, the mechanistic role of Nrf2 and hormones in nNOS mediated gastric emptying remain inconclusive.

In the past decade, it has been shown that dietary compounds targeting Nrf2 activation and BH_4 can be used therapeutically in reducing the risk of obesity and type 2 diabetes [3,12,13]. *Cinnamonum zeyla-nicum* (cinnamon) is known to be an Nrf2 activator via AKT/JNK pathway and is a popular traditional medicine to treat diabetes [16,17]. Previous studies have shown that supplementation of the cinnamalde-hyde (CNM) bioactive compound present in cinnamon, improved renal function and delayed gastric emptying in the onset of diabetes in male rodents [18,19]. However, the molecular signaling of Nrf2 activation and its mechanistic role on regulating gastric emptying has not been well delineated in female Obese/T2D mice.

Hence, our studies are aimed to investigate the mechanistic role of cinnamaldehyde, as an Nrf2 activator in gastric nitrergic relaxation and solid gastric emptying in high fat diet-induced (HFD) obesity and type 2 diabetic gastroparesis using adult female homozygous *Nfe2l2*$^{-/-}$ (B6.129X1-Nfe2l2 tm1Ywk/J, KO) and their wild-type (WT) littermates (*Nfe2l2*$^{+/+}$) mice. Our results demonstrate that CNM restored GE in WT-HFD but not in *Nfe2l2*$^{-/-}$ female mice.

2. Materials and methods

2.1. Animals

The Institutional Animal Care and Use Committee (IACUC) at Meharry Medical College (MMC) approved all animal experiments in this study. Adult (8–9 week-old) female homozygous *Nfe2l2*$^{-/-}$ mice (B6.129X1-Nfe2l2 tm1Ywk/J, KO) and their wild-type (WT) littermates were purchased from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, ME). All animals were allowed access to food and water ad libitum. The diet consisted of either a normal diet (ND, 6.2% energy as fat, Teklad Global 2018, Teklad Diets, Madison WI) or a high fat diet (HFD, 70% energy as fat, 19% protein, and 11% carbohydrate, HFD; 5SSV, TestDiet, St. Louis, MO).

2.2. Experimental design

Mice were randomly assigned to one of six treatment groups (n = 6–8 per group): (i) wild type-normal diet (WT-ND); (ii) wild type-high fat diet (WT-HFD); (iii) wild type-high fat diet + cinnamaldehyde (WT-HFD + CNM, 50 mg/kg); (iv) knock out-normal diet (KO-ND); (v) knock out-high fat diet (KO-HFD); (vi) knock out-high fat diet + cinnamaldehyde (KO-HFD + CNM). Previous studies have shown that 50 mg/kg b.w CNM is effective to restore renal function in the diabetic mouse [19]. Therefore, we have selected the similar CNM dose regimen for the current study. Cinnamaldehyde was prepared in mineral oil (carrier solution) and administered intraperitoneally (i.p.), three times a week for twelve weeks. At week 12, mice from all the groups were sacrificed by CO_2 asphyxiation. Stomach tissues were snap frozen and stored at −80 °C.

2.3. Fasting blood glucose, glucose tolerance test and insulin tolerance test

Fasting blood glucose levels were monitored every alternate week for the duration of the study period using standard protocol [20]. During the 10th week of the study, intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) was carried out at 11th was performed as reported previously [20]. Data were expressed as the absolute values of blood glucose concentrations.

2.4. Assessment of estrus cycle

The progression of two consecutive estrus cycles (Proestrus, estrus, metaestrus and diestrus) were assessed by vaginal smears after female mice were fed on ND or HFD for 12 weeks [21]. Vaginal smears were collected with a smooth edged glass Pasteur pipette filled with 10 μl of normal saline (0.9% NaCl). The vaginal smear containing cells were placed on an untreated glass microscopic slide and viewed at 20× and 40× magnification to record the stage of estrus cycle.

2.5. Obesity markers, 17β-estradiol (E_2) and serum nitrite assay

Animals were sacrificed by CO_2 asphyxiation and blood was drawn immediately by cardiac puncture. Serum was separated from the blood by centrifugation (2000×g at 4 °C, 15 min). Aliquots of serum was stored in − 80 °C until used for biochemical analysis. Serum cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-10, leptin, Insulin-like growth

factor 1 (IGF-1), IL-6, IL-1a, IL-1b, and Monocyte chemoat-tractant protein-1 (MCP-1)] were measured by multiplex Mouse Cytokine 8-plex Assay (Signosis, Santa Clara, CA). The absorbance at 450 nm was recorded using a micro-plate reader (BioTek, Winooski, VT). Nitrite levels in serum were analyzed as total nitrite (metabolic byproduct of NO) according to the manufacturer's protocol (Bio vision, Milpitas, CA). Serum 17 beta-estradiol (E_2) levels were assayed using an ELISA kit (Enzo Life Sciences, Farmingdale, NY) as per the manufacturer's instructions. The absorbance at 405 nm was recorded using a micro-plate reader (BioTek, Winooski, VT).

2.6. Solid gastric emptying studies

Solid gastric emptying studies were performed as described previously [10]. Briefly, after fasting overnight (providing water), known amounts of food were fed to the animals for 3 h. At the end of 3 h, the remaining food was weighed in order to establish the amount of food intake. Animals were fasted for 2 h without food and water and then sacrificed. Gastric tissue were collected and the weight of the whole stomach was recorded. The rate of gastric emptying was calculated according to the following equation: gastric emptying (% in 2 h) = (1 − gastric content/food intake) × 100.

2.7. Organ bath studies

As reported earlier, electric field stimulation (EFS)-induced non-adrenergic non-cholinergic relaxation (NANC) was studied in WT (n = 4/group) circular gastric antrum neuromuscular strips [3,9–11]. To investigate the in-vitro effect of CNM (100 μM; 30min) under normoglycemic or hyperglycemic conditions (50 mM, 30 min), muscle strips were pre-incubated and NANC dependent nitrergic relaxation (nNOS function) was determined at 2Hz [3,9–11]. In a separate set of experiments, to investigate the role of Nrf2 signaling pathways, muscle strips were preincubated with SP600125 (10 μM), an inhibitor of ERK/JNK for 30 min in the presence or absence of CNM. The NO dependence of nitrergic relaxation was confirmed with NG-nitro-L-arginine-methyl ester (L-NAME, 100 μM, 30 min). Comparisons between groups were performed by measuring the area under the curve (AUC/mg of tissue) 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) = AUC/mg of tissue".

2.8. nNOSα dimerization in gastric neuromuscular tissue

nNOSα monomer and dimer expressions were quantified by Western blotting via low temperature (LT)-polyacrylamide gel electrophoresis (PAGE) in gastric antrum homogenates as described previously (3, 8, 9). A polyclonal antibody (N-terminal) specific to nNOSα (1:500, Thermo Fisher Scientific, Waltham, MA) and anti-rabbit IgG conjugated with horseradish peroxidase (1:10000, Sigma Chemical, St. Louis, MO) were used as the primary and secondary antibodies, respectively.

2.9. Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Total tissue RNA was isolated from the mice gastric neuromuscular tissues by a single-step guanidine thiocyanate method using Trizol (Invitrogen, CA). The quality of RNA was

determined by NanoDrop™ (Thermo Fisher Scientific). The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used to synthesize cDNA. RT-qPCR amplification was performed using the Syber-Green (Bio-Rad, Hercules, CA) method. Expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. Relative amounts of mRNA were normalized to p-actin. Primers used for quantitative analysis are listed in Table 1. All studies were performed in the MMC Molecular Core Laboratory.

2.10. Western blot analysis

Proteins from gastric antrum neuromuscular tissue lysates were separated by SDS-PAGE. The membrane was immunoblotted with polyclonal Nrf2, Kelch-like ECH-associated protein 1 (Keap-1), super-oxide dismutase 1 (SOD 1), catalase (CAT) and GTP cyclohydrolase 1 (GCH-1) from Santa Cruz (CA), *p*-ERK, *p*-JNK, p38/MAPK (Cell Signaling, Danvers, MA), nNOS α (N— terminal, Abcam, Cambridge, MA), primary antibody and anti-rabbit IgG conjugated with horseradish peroxidase as secondary antibody (1: 10,000, Sigma Chemicals, St. Louis, MO). All primary antibodies were probed as per the manufacturer's recommendation (1:500 or 1:1000) dilutions respectively. Binding of antibodies to the blots was detected using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's instructions. Stripped blots were re-probed with β-actin-specific polyclonal antibodies (Sigma Chemical) to enable normalization of signals between samples. Band intensities were analyzed using ImageQuant LAS 500 (GE Health Sciences, Pittsburgh, PA).

2.11. Statistical analysis

Data were presented as the mean ± standard error (SE). Statistical comparisons between groups were determined by the Student's *t*-test or Tukey's test after one-way Analysis of Variance (ANOVA), using the GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA). A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of cinnamaldehyde-on body weight and blood glucose levels

High fat diet (HFD) group in both Nrf2 KO (*Nfe2l2*$^{-/-}$) and wild type mice showed a significant ($p < 0.05$) higher body weight when compared to the normal diet (ND) fed group (Table 2). Supplementation of CNM significantly lowered total body weight (27.1 ± 0.9 g, $p < 0.01$) when compared to HFD group in both WT and Nrf2 KO mice. In addition, CNM significantly attenuated elevated blood glucose levels in both WT and Nrf2 KO mice those fed with HFD (Table 2).

3.2. Cinnamaldehyde reverses the effects of HFD on estrus cycle in a Nrf2 dependent manner

As depicted in Fig. 1, HFD fed mice showed abnormalities in cycling phases; progression and cycle length (7–8 as compared to 4–5 in ND fed mice, $p < 0.05$). These abnormalities include; elongation of phases (e.g. diestrus for 4 consecutive days), skipping of phases (e.g. metestrus), or a combination of both indicating disruption in the reproductive cycle (Fig. 1). CNM significantly restored cycle predominance to the estrus/meta estrus phases (Fig. 1A –

C, 1G) as well as decreased cycle length (6 days, $p < 0.05$) in WT mice, but not in Nrf2 KO mice (8 days) (Fig. 1D – F, 1H). While glucose tolerance was apparent in HFD group, mice with CNM supplementation significantly improved glucose tolerance (Fig. 1Iand J). In concurrence with this observation, ITT data indicates that CNM maintained insulin sensitivity at the normal level similar to that of mice fed on a normal diet (Fig. 1K & L).

3.3. Cinnamaldehyde restored serum obesity markers, 17β-estradiol (E_2) and nitrite levels

Serum leptin concentrations exhibited very strong correlation with body weight (Fig. 2A). As shown in Fig. 2B–G, obesity marker levels were significantly ($p < 0.05$) higher in HFD mice compared to ND mice. CNM significantly ($p < 0.05$) normalized obesity markers, in WT HFD-fed mice but not in Nrf2 KO mice. As shown in Fig. 2Hand I, serum E_2 levels were elevated in HFD-fed groups (WT and KO) in comparison to ND groups whereas serum nitrite levels were significantly ($p < 0.05$) reduced in HFD-fed groups (WT and KO) (Fig. 2K & L). Supplementation of CNM normalized both E_2 and nitrite levels in WT-HFD but not in KO-HFD group.

3.4. Nrf2 is required for cinnamaldehyde-mediated reversal of HFD-induced delay of gastric emptying in wild type mice

As shown in Fig. 3, HFD fed mice displayed a significant decrease (38%) in solid GE compared to ND fed mice (77.4 ± 3.4% vs 29.3 ± 4.9%). CNM normalized delayed gastric emptying significantly (75.7 ± 10.0% vs 29.3 ± 4.9%, $p < 0.01$) compared to WT-HFD but not in Nrf2 KO-HFD fed mice (Fig. 3Aand B).

3.5. Cinnamaldehyde restored gastric ERK, JNK and p38/MAP signaling pathways, Keap-1, phase II detoxifying, GSK-3β and AhR expression

Shen et al., demonstrated that Nrf2 transactivation required ERK and JNK signaling [22]. Having shown that CNM-mediated rescue of GE requires Nrf2, we next investigated if HFD affected these signaling pathways. Our data in Fig. 4A and B shows that feeding HFD to WT and Nrf2 KO female mice resulted a statistically significant decrease ($p < 0.05$) in both ERK and JNK phosphorylation in gastric neuro-muscular tissue. Supplementation of CNM reversed HFD induced decrease of these pathways in WT but not in KO female gastric tissue.

In addition, our results depicted in Fig. 4C demonstrate that HF diet suppresses Nrf2, induces Keap-1 expression, whereas CNM increases Nrf2 protein levels and reduces the protein expression of Keap-1, the negative regulator of Nrf2 phase II antioxidant enzyme genes. Finally, we also have noticed that CNM attenuated decreased expression of Nrf2 targeted gastric phase II antioxidant enzyme genes; CAT and SOD 1 ($p < 0.05$) in WT but not in Nrf2 KO mice. (Fig. 4 D–K).

Since GSK-3β is inhibited by phosphorylation at Ser9 by Ser/Thr protein kinases via AKT, it has been suggested that Nrf2 might be up-regulated through activation of AKT and inactivation of GSK-3 [23]. The results indicate that supplementation of CNM phosphorylate GSK-3β in WT Obese/T2D gastric neuromuscular tissues (Fig. 4L). In addition, activation of hydrocarbon receptor (AhR) is linked to major diseases, including

obesity, and also evidenced that activated AhR inhibits the expression of E_2 induced genes [24–26]. We investigated the expression of AhR in WT-HFD and Nrf2 KO mice gastric tissue. The results shows that supplementation of CNM substantially attenuated elevated expression of gastric AhR in WT-HFD mice but not in Nrf2 KO mice fed with HFD (Fig. 4M).

3.6. Effect of cinnamaldehyde on gastric estrogen receptor expression

Previous studies have shown that p^{38}/MAPK and AhR may contribute to E_2 mediated ER binding and function [26,27]. As shown in Fig. 5, both gastric ERα and ERβ mRNA & protein expressions were suppressed in HFD fed mice. In addition, CNM supplementation significantly ($p < 0.5$) upregulated both ER's expression only in WT-HFD but not in Nrf2 KO mice (Fig. 5 A–H).

3.7. Cinnamaldehyde normalized gastric nNOSα mRNA & protein expression and dimerization in wild type but not Nrf2 KO mice

As depicted in Fig. 6, nNOSα expression was significantly ($p < 0.05$) reduced in HFD fed mice, whereas CNM supplementation enhanced the expression of nNOS α expression in WT mice but not in Nrf2 KO mice. To measure whether the decrease in the nNOSα was due to altered nNOS dimer (an indirect measurement for enzyme activity) levels, we performed the dimerization study by LT-PAGE gel. As shown in Fig. 6E, a significant ($p < 0.01$) decrease in the dimer/monomer ratio was noticed in HFD fed mice in both WT and Nrf2 KO mice. Whereas, supplementation of CNM significantly restored dimerization on WT + HFD but not in Nrf2 KO obese/T2D mice. (Fig. 6E and F).

3.8. Cinnamaldehyde attenuated diabetes induced decrease in gastric GTP cyclohydrolase I (GCH-1) expression

GCH-1 play a role in BH_4 biosynthesis via de novo pathway [28]. As depicted in Fig. 6 G – J, HFD significantly ($p < 0.05$) reduced GCH-expression in WT but not in Nrf2 KO. In addition, supplementation of CNM restored GCH-1 expression.

3.9. Cinnamaldehyde restored diabetes-induced impairment of gastric nitrergic relaxation in in vitro

As shown in Fig. 7A, a statistically significant ($p < 0.05$) decrease in nitrergic relaxation was observed in neuromuscular tissues exposed to hyperglycemic conditions when compared with normal glucose group. Further, pre-incubation with CNM (100 μM) resulted in attenuation of hyperglycemia impaired gastric nitrergic relaxation (Fig. 7A). As shown in Fig. 7B, inhibition of JNK/ERK signaling pathway with SP600125 significantly impaired nitrergic relaxation of gastric neuromuscular tissue. Furthermore, pre-incubation with CNM restored gastric nitrergic relaxation suggesting that Nrf2 is playing a critical role in nitrergic neuron function (Fig. 7B).

4. Discussion

Gastroparesis is more prominent in both obese and diabetic patients. Gastric emptying depends on many factors such as autonomic and enteric nerve damage (excitatory and

inhibitory nerves), interstitial cells of Cajal (ICC) malfunction, drastic fluctuations in blood glucose, medications related to incretin and aggravated by a mental factor via autonomic mechanisms [29]. Earlier studies from our laboratory have shown that supplementation of BH_4/sepiapterin restores nNOS mediated gastric emptying by alleviating Nrf2-Phase II enzymes in diabetic rodents [3,13,30,31]. Deletion of Nrf2 gene reduced nitrergic relaxation and delayed gastric emptying [10]. In addition, previous studies identify that Nrf2 pathway is a novel target for management of obesogenesis as well as glucose homeostasis in male mice fed with HFD [20,32]. However, studies are limited to investigate the molecular signaling of Nrf2 activation and its mechanistic role on regulating gastric emptying in female obese/T2D mice. In the current study, by using C57BL/6J WT and Nrf2 KO high-fat diet-fed mouse models, we have demonstrated that activation of Nrf2 attenuated delayed gastric emptying by normalizing (1) body weights, (2) fasting glucose and IPGTT's/ITT, (3) serum E_2, NO, obese markers (4) gastric ERK/JNK/Keap-1 (5) GSK3β, AhR, p38/MAPK, ERα and ERβ, (6) BH_4 (Cofactor of nNOS) biosynthesis enzyme GCH-1 (de novo), (7) nNOSα protein & dimerization and (5) Nrf2 and phase II antioxidant enzymes (Fig. 8). Collectively, our data suggest that activation of Nrf2 by CNM is not only important in bringing glucose homeostasis and normalizing circulatory markers but also regulating normal gastric emptying by restoring altered gastric nNOS function [20].

In addition to cinnamaldehyde, several other activators such as sulforaphane and curcumin activates Nrf2 and trigger various cell-signaling mechanisms in type 2 diabetes [16]. Cinnamaldehyde and its metabolites are well distributed in all the organs such as heart, liver, spleen, lung, kidney, and brain [33]. In this study, we have selected cinnamaldehyde because it exerts its beneficial effects on multiple cell signaling pathways via Nrf2 [33]. Several studies have demonstrated the vital role of various Nrf2 activators in controlling diabetes and its secondary complications [16]. However, none of the above studies have investigated the mechanistic role of Nrf2 activators with context to diabetic gastroparesis. Recent studies have shown significant alterations in circulatory inflammatory cytokines in gastroparesis patients [34]. Inflammation is commonly seen in all obesity and diabetic humans and in animal models that can be primarily initiated by nutrient overload [35]. Our current data demonstrated that supplementation of CNM, neutralized pro-inflammatory cytokines as well as leptin suggesting an effective strategy for improving glucose tolerance and insulin sensitivity in HFD induced obesity. Many studies have suggested that Nrf2-deficient mice are more prone to cell death, inflammation and genotoxic effects induced by oxidants as well as electrophiles [6,30,36,37]. The results from WT and Nrf2 KO mice in this study further indicate that activation of Nrf2 by CNM plays a protective role against inflammation and oxidative stress and normalizes altered gastric emptying.

Sex steroid hormones mediate their biological actions through their nuclear and cytoplasmic/membrane receptors [38]. Tamir et al. [39], reported that oxidative stress, a well-known consequence of diabetes, differentially regulates the expression of ERα and ERβ in various cell types. Our results demonstrate that estrogen receptors have been reduced in HFD fed mice stomach tissue while serum estrogen levels are elevated. Supplementation of CNM improved gastric estrogen receptors, normalized estrus cycle, serum estrogen and nitrite and accelerated gastric emptying in WT but not in Nrf2 KO fed with HFD. Jasik and Lustig [40], reported that large amount of circulatory estrogen are being released from adipose tissue due

to increased conversion of testosterone in mice fed with HFD. Long-term obesity decreases liver functions by estrogen 2-hydroxylation, which also increases circulatory estrogen levels [41]. Our data suggest that Nrf2 may have a direct or indirect effect on ovarian as well as adipose endocrine system and maintain normal gastric emptying in HFD induced obese/T2D female mice. In addition, these studies may have a direct clinical relevance to address the beneficial role of ovarian estrogens and/or their gastric receptors in diabetic gastroparesis women. Studies are underway to investigate the potential role of estrogens and/or their gastric receptors in NO mediated gastric function.

In addition to demonstrating the therapeutic potential of CNM in suppressing diabetic GP, we have investigated the mechanism by which Nrf2 activation was able to attenuate the delayed GE. Nrf2 signaling pathways regulate several biochemical processes such as gene expression, localization of proteins and its expression, cell cycle, and stress responses [6]. Thus, from a regulatory viewpoint, it is important to delineate the effect of signaling pathways involved in the regulation of CNM mediated Nrf2 activation and phase II enzyme expression. Consequently, the extent of gastric *p*-ERK/*p*-JNK and p38/MAPK kinases was further investigated following CNM treatment in both WT and Nrf2 KO with or without fed with HFD in female mice. The results in this study reveal that CNM increased both ERK/JNK, p38/MAP kinase signaling pathways and phase II protein expression through Nrf2 activation in WT but not in Nrf2 KO fed with HFD in female mice. Our studies for the first time highlight the importance of Nrf2 activation on regulating gastric emptying and underlying mechanisms in HFD female rodents.

Nrf2 is regulated principally by two different mechanisms. First mechanism is by Keap-1 an ubiquitin E3 ligase substrate adapter for a Cullin3/Rbx1-dependent E3 ubiquitin ligase complex; proteasomal degradation of Nrf2 happens by binding of Keap-1 to Nrf2 [42]. The second mechanism is by GSK-3β, which phosphorylates Nrf2 creating a recognition site for β-Transducin Repeat Containing E3 Ubiquitin Protein Ligase (β-TrCP) which leads to Cullin-1/Rbx1-mediated Nrf2 ubi-quitination and its subsequent degradation in the cytosol [43]. Thus GSK-3β is also known to degrade Nrf2 in the nucleus via the β-TrCP-GSK3β axis [44]. Glycogen synthase kinase (GSK) 3β has emerged to be an integration point and acts as a pivotal role in controlling Nrf2 activity. Glycogen synthase kinase-3 is a serine/threonine kinase with important roles in the regulation of glycogen synthesis, protein synthesis, gene transcription, and cell differentiation in various cell types. Impaired ability of insulin to activate glucose disposal and glycogen synthase are linked to GSK-3 overexpression in skeletal muscle of obese rodent models and diabetic humans [45]. Nrf2 might be upregulated through activation of AKT and permanent inactivation of GSK-3 by phosphorylation at Ser9 by Ser/Thr protein kinases [46,47]. In this study, we have demonstrated that treatment with CNM disrupts the Keap-1/Nrf2 interaction and through the GSK-3β/Nrf2 signaling pathway, provides a double mechanism of activation of Nrf2.

Aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates toxicological responses and has been reported to influence the development of obesity and metabolic disorders [48]. A recent study suggests that modern western diet activates AhR signaling [48]. Previous studies suggest that a cross talk between Nrf2 and AhR exists while Nrf2 controls AhR signaling [49,50]. In addition to interactions with Nrf2 signaling, AhR

also modulates its function on estrogen receptor (ERα and ERβ) and androgen receptor (AR). AhR activation by assembling a ubiquitin ligase complex, $CUL4B^{AHR}$ was shown to promote the ubiquitination and proteasomal degradation of ERs and AR [51,52]. Our data highlight the fact that supplementation of CNM reduced gastric AhR by which enhances ER's expression and protects against diet-induced obesity and its metabolic complications in WT + HFD. In addition, our data shows that CNM activated estrogen receptor dependent p38/MAPK signaling pathway. Previous studies have shown that activation of p38/MAPK known to promote ER receptor efficiency to translocate into the nucleus, which can then bind directly to estrogen response element (EREs). Several lines of evidence suggest that estrogens maintain gastric emptying in both rodents and humans [9]. Shah et al., studies demonstrated that nNOS mediated gastric relaxation is regulated by estrogens [53]. Collectively, the above data suggest activation of Nrf2 by CNM regulate gastric ER and maintain nNOS mediated gastric emptying in female T2D mice.

Neuronal nitric oxide synthase (nNOS) activity represents a critical signaling node for regulating gastric motor function. nNOS catalyzes the formation of nitric oxide (NO), which initiates smooth muscle relaxation. nNOS activity in turn is regulated by tetrahydrobiopterin (BH_4) a cofactor for nNOS dimerization and enzyme activity [13]. Loss of Nrf2 plays a significant role on the BH_4 levels in turn on nNOS function [10]. Our current studies demonstrate that supplementation of CNM attenuated reduced gastric nNOSa and nNOSα dimerization in WT HFD but not in Nrf2 KO HFD mice. In addition, supplementation of CNM restored the protein expression of GCH-1 (de novo enzyme for BH_4 biosynthesis) but not DHFR (salvage) in WT fed with HFD. Finally, our studies demonstrate that CNM restored hyperglycemia induced impairment of gastric nitrergic relaxation via AKT/ERK/JNK mechanism. These results suggest that obesity-induced hyperglycemia contribute to decreased availability of BH_4 and nNOSα uncoupling which results in impaired nitrergic relaxation and delayed gastric emptying. In addition, Xue et al. [54], suggested that GCH-1 activated by Nrf2, increases BH4 and inhibits NOS uncoupling, which reduces the generation of ROS. Collectively, the above data suggest that activation of Nrf2 by CNM enhances the expression of BH_4 enzyme GCH-1, nNOS dimerization and normalizes delayed gastric emptying in obese/T2D female mice.

In conclusion, results obtained in this study showed that CNM effectively prevented HFD-induced obesity and T2D. The possible mechanism involved in controlling diabetic gastroparesis is through modulation of key regulating detoxifying enzymes via Nrf2 mediated gastric ER and nNOS function. This suggests that CNM possess potential therapeutic value for treating and/or management of diabetes and its secondary complications such as gastroparesis. This bioactive compound could be a suitable candidate for use in clinical studies as a pharmaconutritive agent for alleviation of diabetic gastroparesis.

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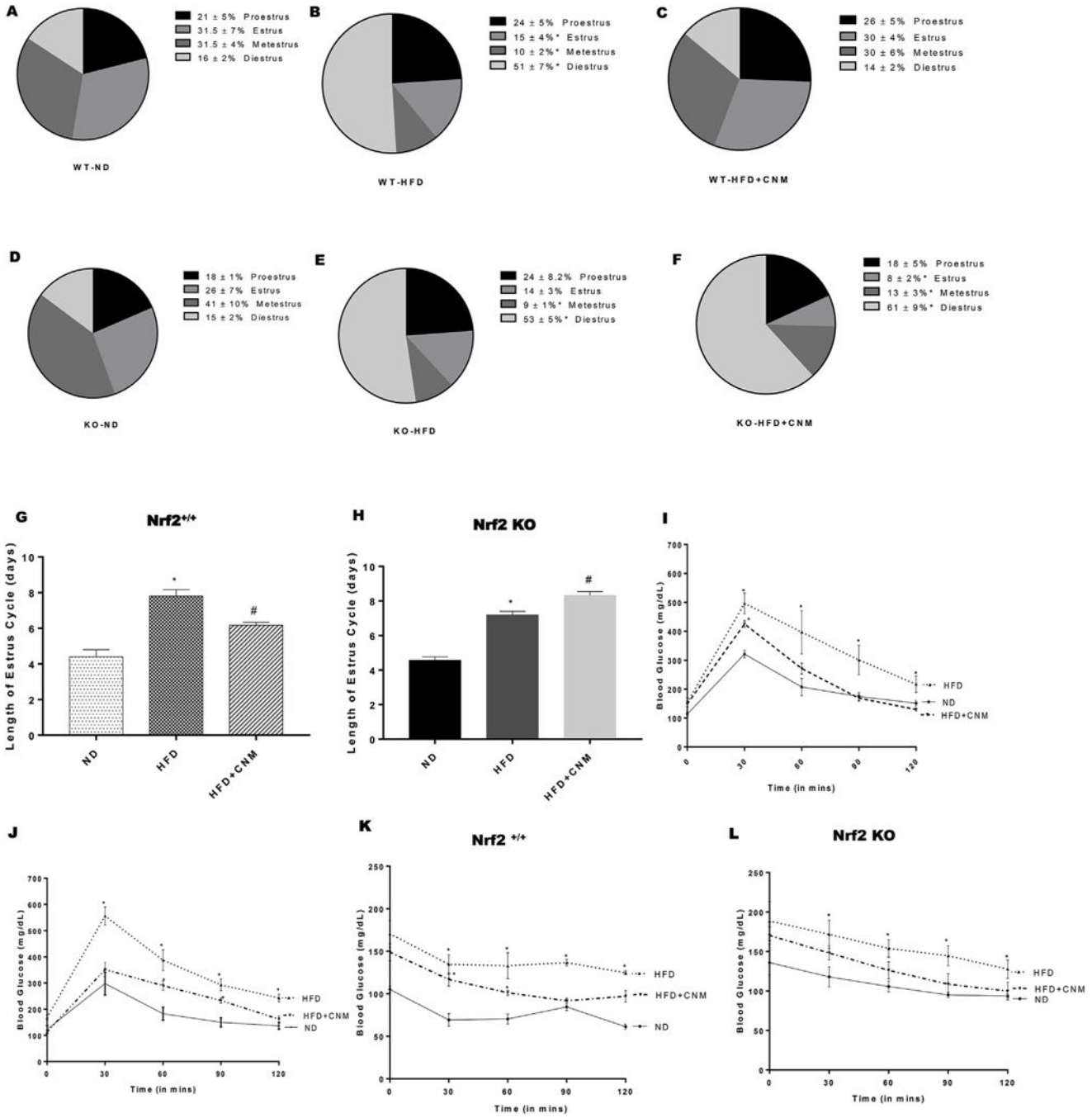

Fig. 1. Effect of high fat diet (HFD) and cinnamaldehyde (CNM) on estrus cycle in wild type (WT) and Nrf2 KO mice. Glucose tolerance and insulin sensitivity in HFD fed mice. Insulin tolerance test (ITT) in wild type (WT) and Nrf2 KO mice.

(A–F) Representation of cycling days (**G-H**). (**I, J**) Intraperitoneal glucose tolerance test (IPGTT); profile of blood glucose concentration versus time; (K, L) profile of blood glucose concentration (percentage of initial value) as a function of time upon intraperitoneal injection of insulin. Data were analyzed using one way ANOVA by using graph pad prism software. Data are means ± SEM (n = 6). *P < 0.05 compared with normal diet (ND) mice; #P < 0.05 compared to HFD mice.

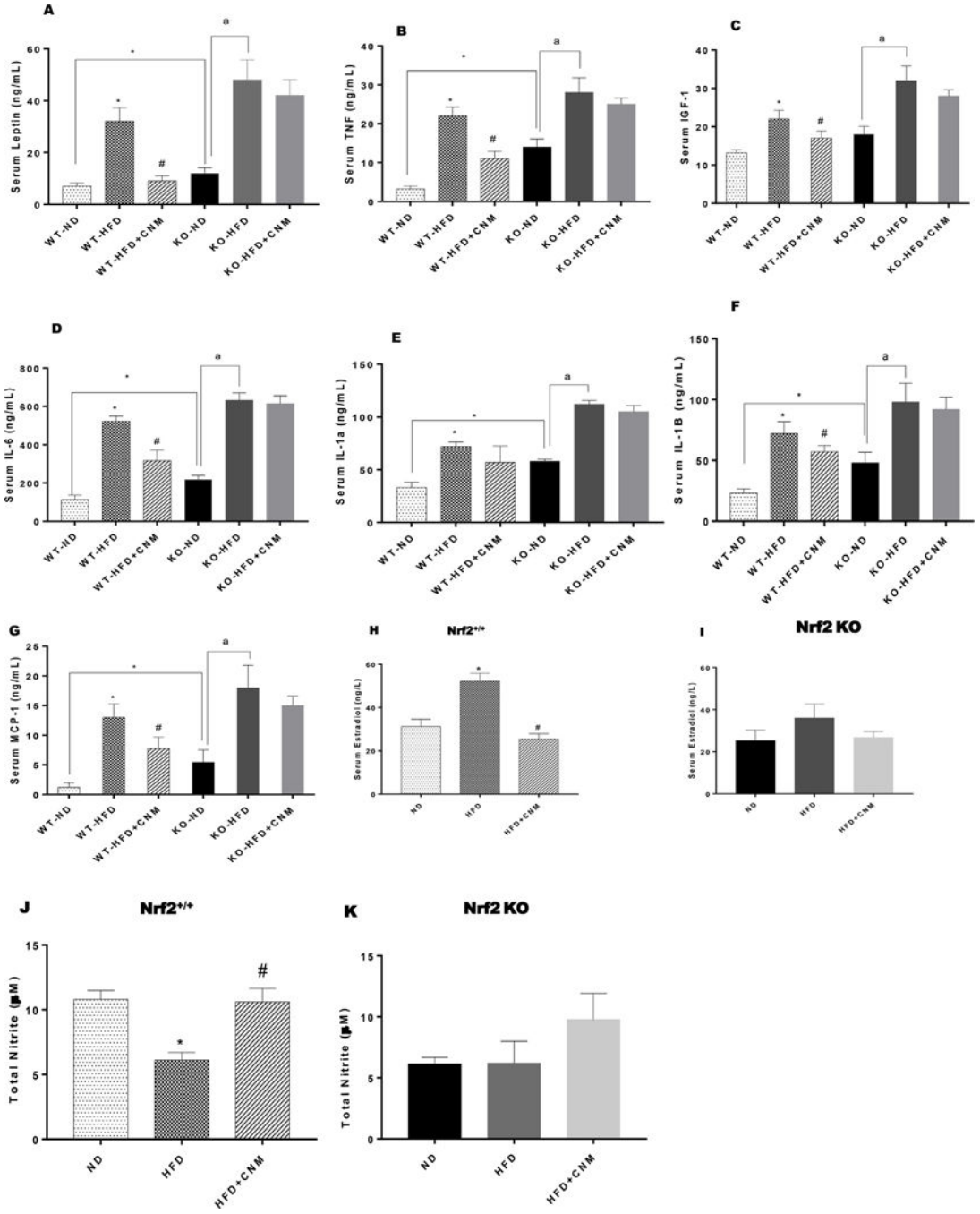

Fig. 2. Effect of cinnamaldehyde (CNM) on serum obesity markers, 17β-estradiol and nitrite levels.

(**A-G**) serum obesity markers; (H-I) serum estradiol levels and (J-K) serum total nitrite (NO) levels. Data were analyzed using one way ANOVA by using graph pad prism software. Data are means ± SEM (n=6). *P < 0.05 compared with ND mice; #P < 0.05 compared to HFD mice.

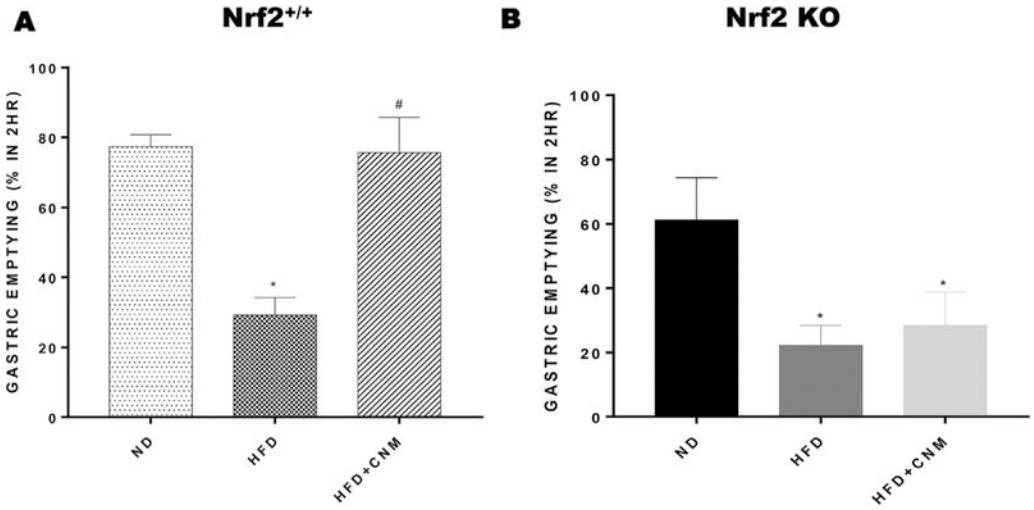

Fig. 3. Effect of cinnamaldehyde (CNM) on solid gastric emptying in wild type (WT) and Nrf2 KO high fat diet (HFD) fed female mice.

A group of WT and Nrf2 KO female mice were fed with normal diet (ND). Data were analyzed using one way ANOVA by using graph pad prism software. Data are means ± SEM (n = 6). *P < 0.05 compared with ND mice; $^{\#}$P < 0.05 compared to HFD mice.

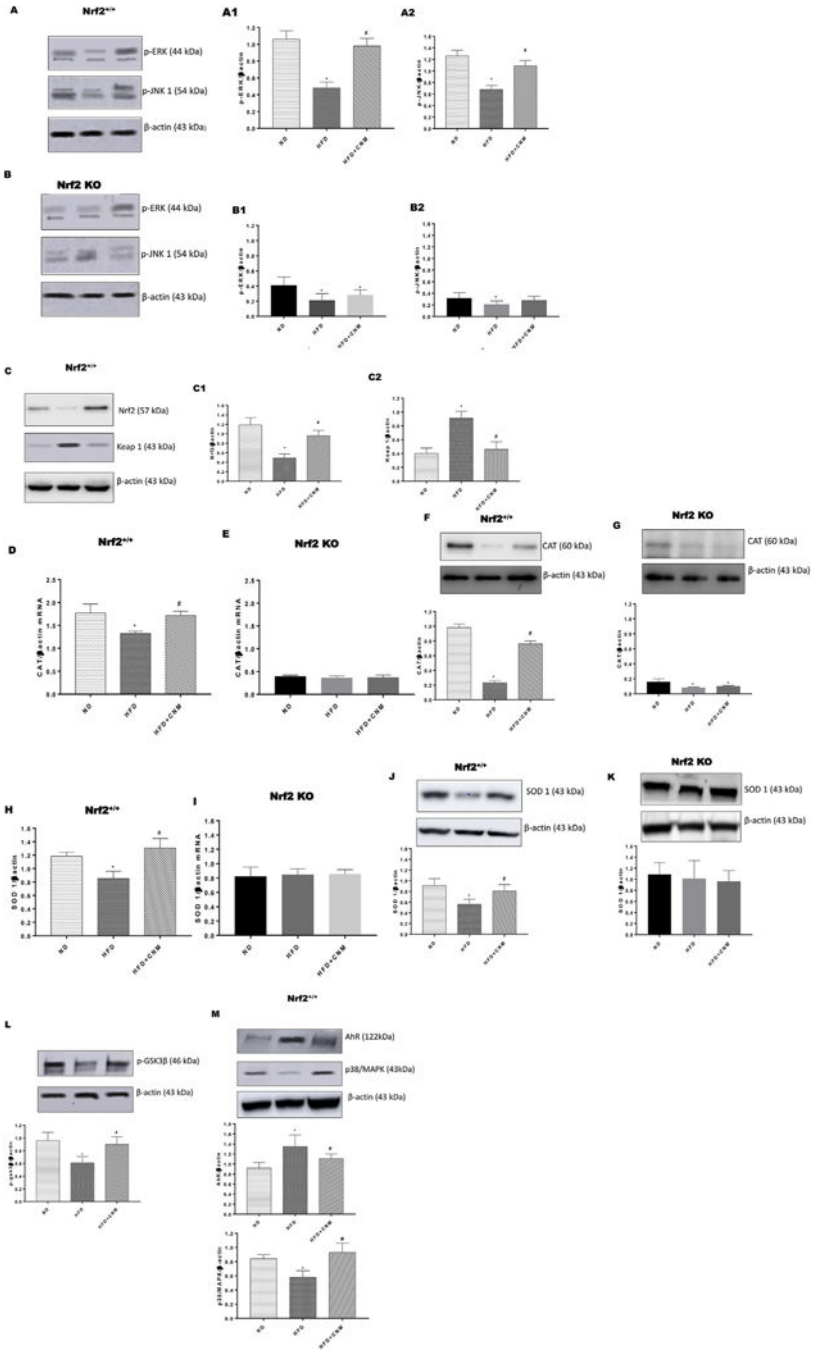

Fig. 4. Effect of cinnamaldehyde (CNM) on protein expressions of wild type (WT) and Nrf2 KO female mice in gastric neuromuscular tissues.

Representative immunoblot and densitometry analysis data for (**A1–2**) *p*-ERK/JNK protein expression in WT, (**B1–2**) *p*-ERK/JNK protein expression in Nrf2 KO, (**C1–2**) Nrf2/KEAP-1 protein expression in WT, (**D**) CAT mRNA expression in WT, (**E**) CAT mRNA expression in Nrf2 KO, (**F**) CAT protein expression in WT, (**G**) CAT protein expression in Nrf2 KO, (**H**) SOD 1 mRNA expression in WT, (**I**) SOD 1 mRNA expression in Nrf2 KO, (**J**) SOD-1 protein expression in WT, (**K**) SOD-1 protein expression in Nrf2 KO (**L**) *p*-

GSK3β protein expression and (**M**) AhR/p38 MAPK protein expression in WT female mice gastric neuromuscular tissue. Stripped blots were re-probed with β-actin. Data were normalized with housekeeping gene or protein (β-actin). Bar graphs showed a ratio of target gene or protein with β-actin. Data were analyzed using one way ANOVA by using graph pad prism software. Values are mean ± SE (n = 4). *$p < 0.05$ compared with ND; #p < compared with HFD fed mice.

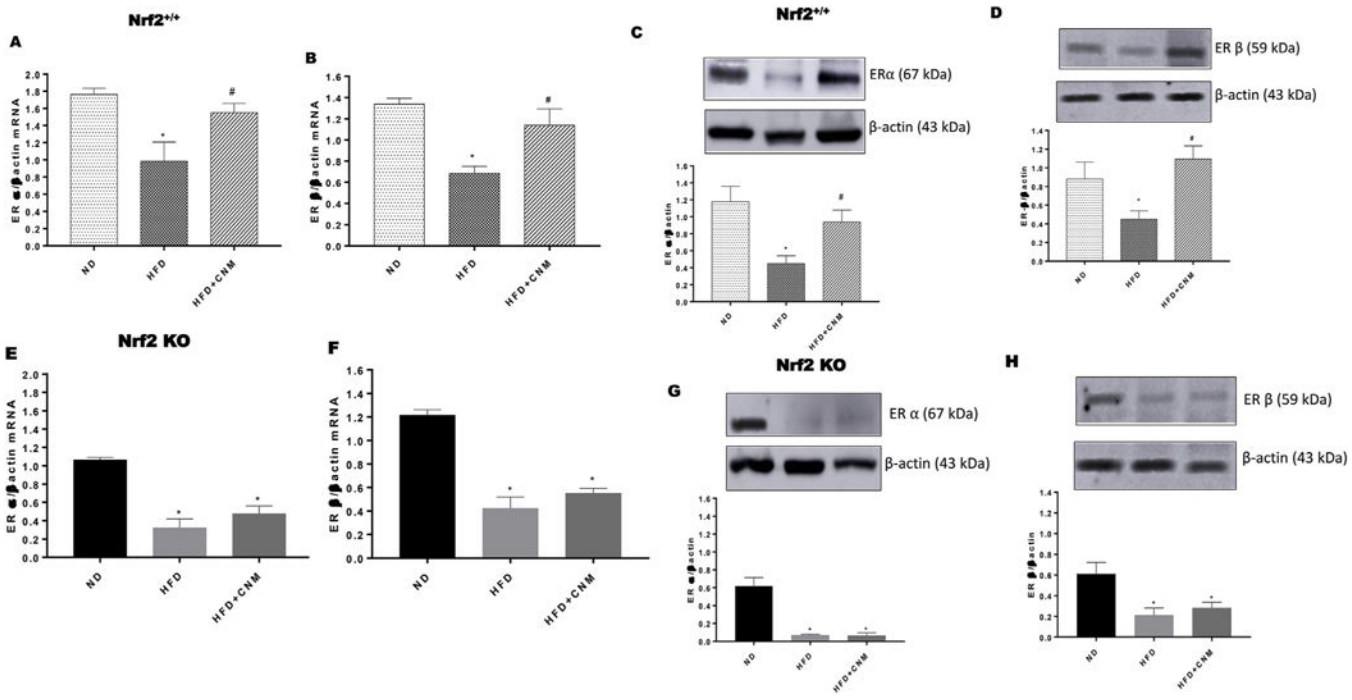

Fig. 5. Effect of cinnamaldehyde (CNM) on mRNA and protein expressions of estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) in wild type (WT) and Nrf2 KO female mice gastric neuromuscular tissues.

Representative immunoblot and densitometric analysis data for (**A**) ER α mRNA expression in WT, (**B**) ER α mRNA expression in WT, (**C**) ER α protein expression in WT, (**D**) ER β protein expression in WT, (**E**) ER α mRNA expression in Nrf2 KO, (**F**) ER β mRNA expression in Nrf2 KO, (**G**) ER α protein expression in Nrf2 KO, (**H**) ER β protein expression in Nrf2 KO in female mice gastric neuromuscular tissue. Stripped blots were re-probed with β-actin. Data were normalized with housekeeping gene or protein (β-actin). Bar graphs showed a ratio of target gene or protein with β-actin. Data were analyzed using one way ANOVA by using graph pad prism software. The values are mean ± SEM (n = 4). *p < 0.05 compared with control group; #p < compared with the diabetic group.

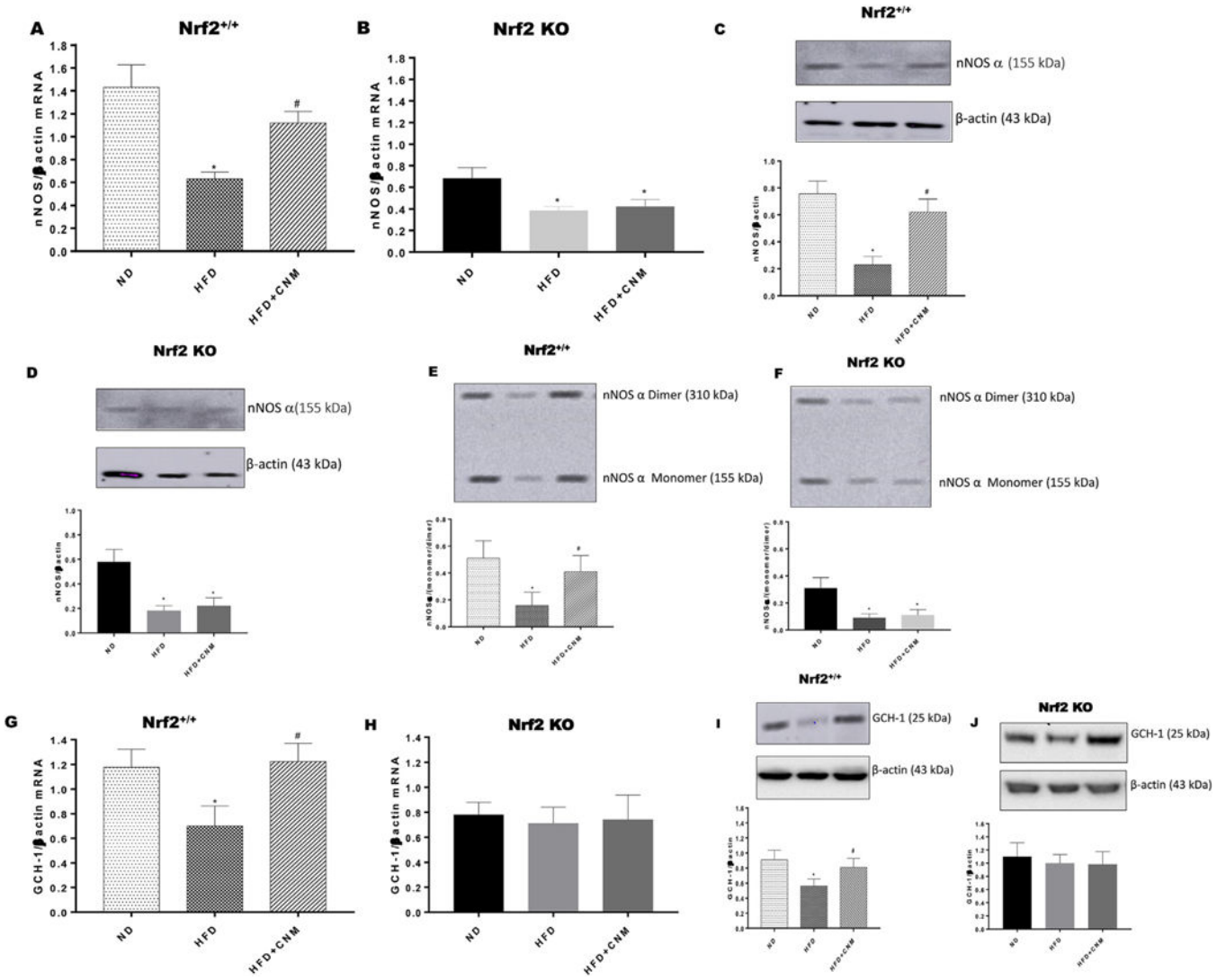

Fig. 6. Effect of cinnamaldehyde (CNM) on mRNA and protein expression of nNOS α.

(**A-D**), nNOSα dimerization (**E, F**) and GCH-1 (**G-J**) in WT and Nrf2 KO female mice gastric neuromuscular tissues. Representative immunoblot and densitometric analysis data for (**A**) nNOSα mRNA expression in WT; (**B**) nNOSα mRNA expression in Nrf2 KO, (**C**) nNOSα protein expression in WT; (**D**) nNOSα protein expression in Nrf2 KO, (**E**) nNOSα dimerization in WT and (**F**) nNOSα dimerization in Nrf2 KO; (**G**) GCH-1 mRNA expression in WT; (**H**) GCH-1 mRNA expression in Nrf2 KO; (**I**) GCH-1 protein expression in WT and (**J**) GCH-1 protein expression in Nrf2 KO in female mice gastric neuromuscular tissue. Stripped blots were re-probed with β-actin. Data were normalized with housekeeping gene or protein (β-actin). Bar graphs showed a ratio of target gene or protein with β-actin. Data were analyzed using one way ANOVA by using graph pad prism software. The values are mean ± SEM (n = 4). *p < 0.05 compared with control group; #p < compared with the diabetic group.

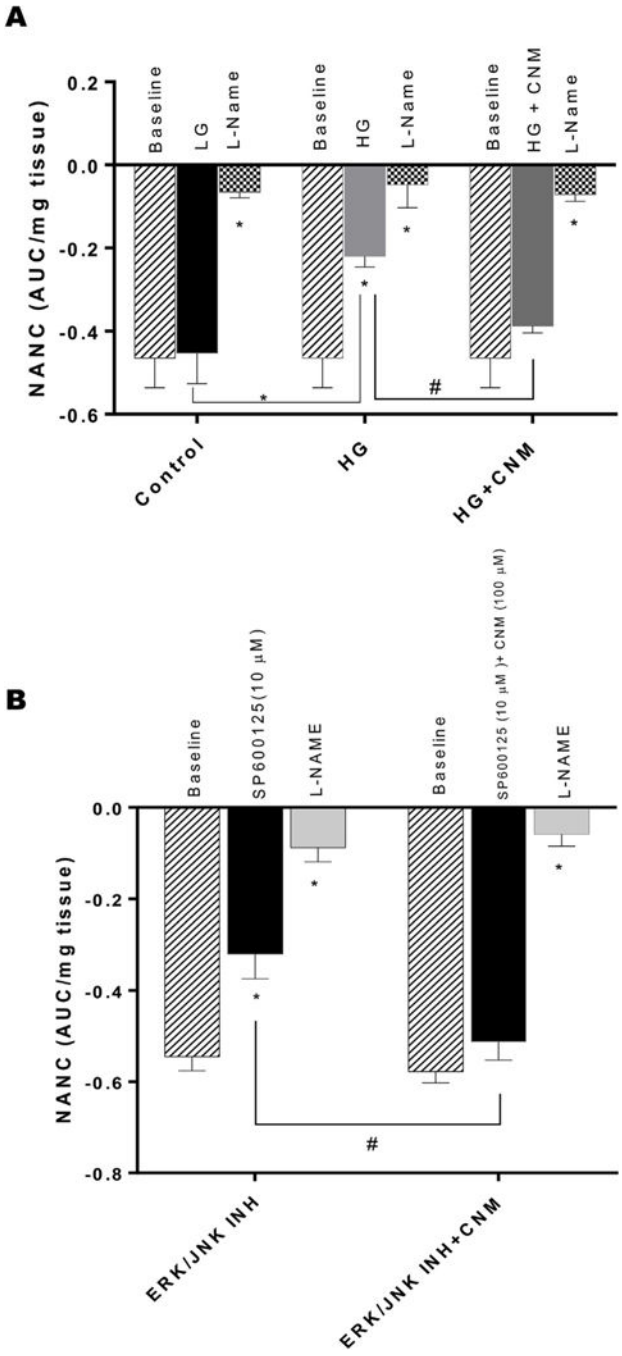

Fig. 7. Nitrergic relaxation of *in-vitro* following electric field stimulation (EFS (2Hz) in circular gastric antrum strips.

The nitric oxide (NO) dependence of the NANC relaxations was confirmed by preincubation (30 min) with the NO inhibitor nitro-L-arginine methyl ester (L-NAME; 100 μM). (**A**) the absence (time control), or in the presence of either high glucose (50 mM) and preincubation of CNM (100 μM) and (**B**) effect of SP600125 for 30 min and then challenged with 100 μM cinnamaldehyde. Data were analyzed using one way ANOVA by using graph pad prism

software. The values are mean ± SE (n = 4), *p < 0.05 compared with the response in the absence of L-NAME.

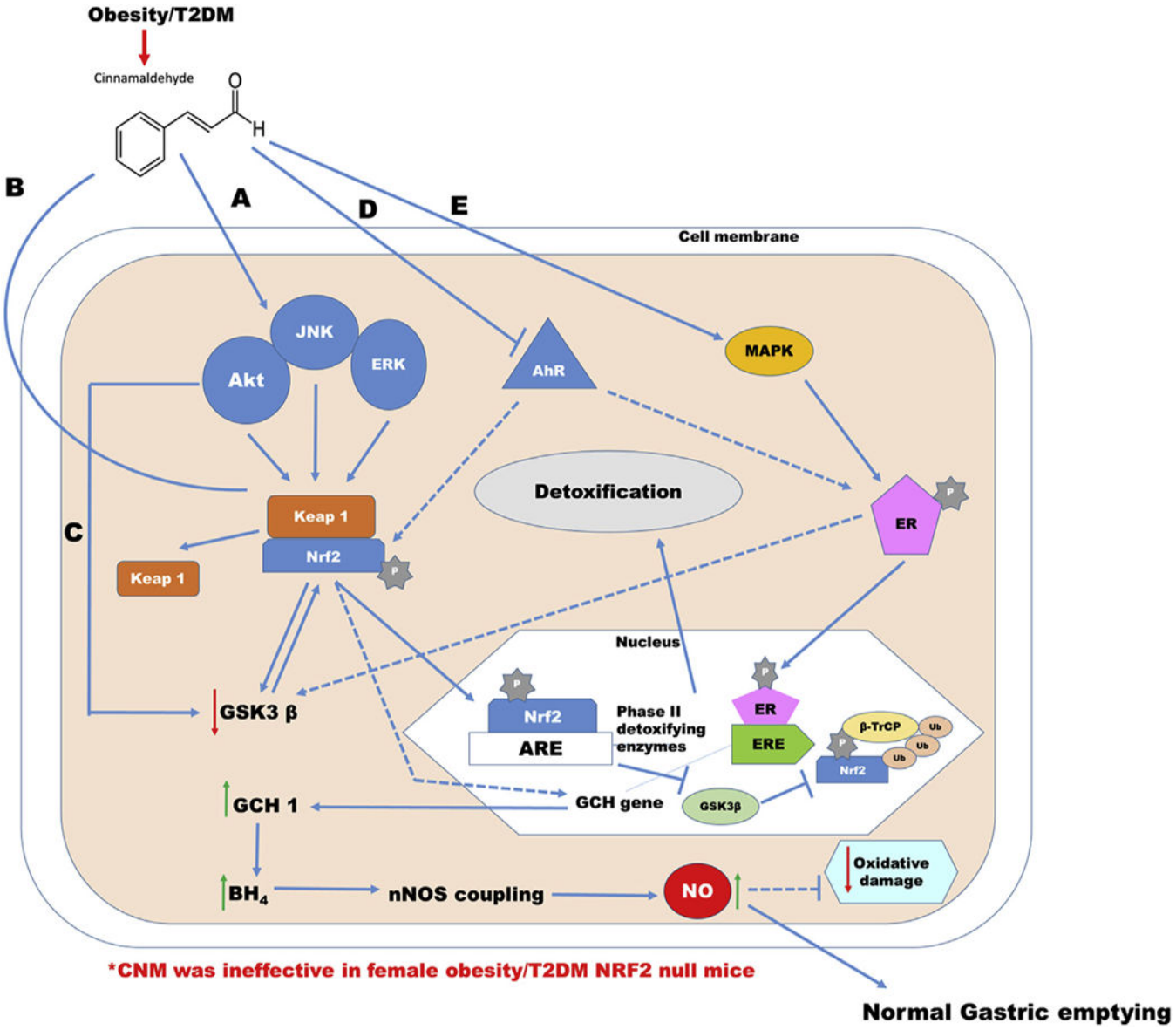

Fig. 8. Schematic illustration depicting the effects of cinnamaldehyde (CNM) on mechanistic signaling of Nrf2 in nNOS mediated gastric motility and gastric emptying in obesity/T2D female mice.

Supplementation of CNM restores delayed gastric emptying via (1) ERK/JNK/Keap-1 (2) GSK3β, MAPK, AhR, ERα and ERβ, (3) BH_4 (Cofactor of nNOS) biosynthesis enzyme GCH-1 (de novo), (4) nNOSα protein & dimerization and (5) Nrf2 and phase II antioxidant enzymes in WT Obese/T2D but not in Nrf2 KO female mice. The solid connections indicate the complexes are tethered through protein-protein interactions to a transcription factor complex that connects the gene promoters to execute transcription of target genes. The dotted connections indicate possible interactions between the proteins. The reversible arrows indicate possible cross talk between the proteins. Arrow indicates activation, whereas bar indicates inhibition. ↑ or ↓marks in the parenthesis indicate increase or decrease levels, respectively

Table 1

Primers used for quantitative real-time PCR.

Gene	Forward	Reverse
SOD 1	5 ′-CGGTTGAGATAGACAGG-3 ′	5 ′-TTAAGTGGTCTTGCACTCG-3 ′
CAT	5 ′-GCGGATTCCTGAGAGAGTGGTAC-3 ′	5 ′-GCCTGACTCTCCAGCGACTGTGGAG-3 ′
nNOS α	5 ′—CCCAACGTCATTTCTGTCCGT-3 ′	5 ′-TCTACCAGGGGCCGATCATT-3 ′
GCH-1	5 ′-GAGCATCACCTTGTTCCATTTG −3 ′	5 ′-GCCAAGTTTACTGAGACCAAGGA −3 ′
ER α	5 ′—CCCGCCTTCTACAGGTCTAAT-3 ′	5 ′-CTTTCTCGTTACTGCTGGACAG-3 ′
ER β	5 ′-CTGTGATGAACTACAGTGTTCCC	5 ′-CACATTTGGGCTTGCAGTCTG-3 ′
β-actin	5 ′-TGGAATCCTGTGGCATCCATGAAAC-3 ′	5 ′-TAAAACGCAGCTCAGTAACAGTCCG-3 ′

Table 2

Body weight, blood glucose and insulin levels in WT and Nrf2 KO female mice at 12 weeks.

	Body Weight (g)	Blood Glucose (mg/dL)
WT-ND	24.2 ± 1.12	119 ± 5.06
WT-HFD	37.19 ± 0.99*	274 ± 15.57*
WT-HFD + CNM	27.11 ± 0.89#	152 ± 18.32#
KO-ND	27.98 ± 1.01	136 ± 13.42
KO-HFD	45.02 ± 3.15*	291 ± 14.78*
KO-HFD + CNM	29.52 ± 0.64#	160 ± 13.95#

Results are expressed as mean ± SEM.

* $p < 0.05$ compared to normal diet,

# $p < 0.05$ compared to High fat diet respectively.