

Role of Sex Hormones And Their Receptors On Gastric Nrf2 And Neuronal Nitric Oxide Synthase Function In An Experimental Hyperglycemia Model

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Abstract

Background: Gastroparesis, a condition of abnormal gastric emptying, is most commonly observed in diabetic women. To date, the role of ovarian hormones and/or gastric hormone receptors on regulating nitrenergic-mediated gastric motility remains inconclusive.

Aim: The purpose of this study is to investigate whether sex hormones/their receptors can attenuate altered Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), neuronal Nitric Oxide Synthase (nNOS) expression and nitrenergic relaxation in gastric neuromuscular tissues exposed to in-vitro hyperglycemia (HG).

Methods: Gastric neuromuscular sections from adult female C57BL/6J mice were incubated in normoglycemic (NG, 5mM) or hyperglycemic (30 mM or 50 mM) conditions in the presence or absence of selective estrogen receptor (ER) agonists (ER α /PPT or ER β : DPN); or non-selective sex hormone receptor antagonists (ER/ICI 182,780, or progesterone receptor (PR)/ RU486) for 48 hours. mRNA, protein expression and nitrenergic relaxation of circular gastric neuromuscular strips were assessed.

Results: Our findings in HG, compared to NG, demonstrate a significant reduction in ER, Nrf2, and nNOS expression in gastric specimens. In addition, in-vitro treatment with sex hormones and/or their agonists significantly (* $p < 0.05$) restored Nrf2/nNOS α expression and total nitrite production. Conversely, ER, but not PR, antagonist significantly reduced Nrf2/nNOS α expression and nitrenergic relaxation.

Conclusions: Our data suggest that ER's can regulate nitrenergic function by improving Nrf2/nNOS expression in experimental hyperglycemia.

1. Background

Gastrointestinal (GI) dysfunction occurs in as many as 20-50% of patients with diabetes mellitus (T1/T2DM) [1]. Diabetic gastroparesis is a syndrome of delayed gastric emptying (GE) in the absence of mechanical obstruction of the upper stomach, antrum body, lower pyloric sphincter, and duodenum. Gastroparesis patients often experience symptoms including severe nausea, vomiting, and abdominal pain [2, 3]. Emerging evidence strongly indicate that women and female rodents are likely to experience more severe disease manifestations of gastroparesis compared to males [3, 4]. In fact, women during their reproductive ages, tend to be disproportionately affected by gastroparesis because their stomach motility is slower to begin with, likely due to elevated levels of sex steroid hormones and nitric oxide[5, 6]. Interestingly, women comprise nearly 80% of the patient population, but this predisposition for Gp remains unclear. Moreover, our laboratory has extensively reported nNOS expression and NO-mediated gut relaxation to be the predominant mechanism severely compromised in female rodent models of diabetic gastroparesis[5, 7, 8]. GI dysmotility, in particular, contributes to malnutrition in diabetic patients offering poor glycemic control and oral drug bioavailability among other concerns. Although options exist, current treatment models include dietary modifications, oral drug therapy (antibiotics, dopamine-2 (D2) receptor antagonists), and surgery[9, 10]. However, despite extensive research, drug therapy that can

improve gastric emptying and decrease symptoms, without too many side effects are limited. Since the underlying mechanisms of gastric dysmotility are poorly understood, it is imperative to work with experimental hyperglycemic conditions to establish translational relevance.

Gastric motility is a highly coordinated activity of smooth muscle contraction and relaxation originating from the enteric nervous system within the stomach and intestine. Gastric function is largely regulated by an (1) excitatory (cholinergic) and inhibitory (nitric oxide (NO)/nitroergic) neurotransmitters working directly on smooth muscles or electrical signals that originate from the interstitial cells of Cajal (ICCs) [11]. Nitroergic signaling, the principal non-adrenergic, non-cholinergic (NANC) inhibitory mechanism in the gastrointestinal tract, plays a critical role in the control of gastric accommodation and pyloric relaxation. Several lines of evidence suggest that loss of Interstitial Cells of Cajal (ICC), neuronal nitric oxide synthase (nNOS) function, and elevated oxidative stress are hallmarks of diabetic gastroparesis [5, 11, 12]. nNOS produces nitric oxide (NO), a neuronal messenger with diverse functions throughout the body, and potent regulator of smooth muscle relaxation. nNOS is expressed as four splice variants: nNOS α , β , λ and μ [13]. Results from our in-vivo studies have demonstrated that nNOS-mediated gastric motility is greater in healthy female compared to male rodents. Furthermore, diabetes induction impaired nNOS α activity in female, but not in male in stomach and duodenum [7, 14]. Other studies have shown that supplementation of 17 β -estradiol (E_2) increases nNOS expression and nitroergic-mediated gastric motility in ovariectomized rats [15, 16]. Furthermore, nNOS requires an essential cofactor, tetrahydrobiopterin (BH_4) for enzyme activity. BH_4 is produced via two distinct pathways. In de novo biosynthesis, guanosine triphosphate (GTP) is converted into BH_4 by GTP cyclohydrolase I (GCH-1). BH_4 is also produced through oxidation of dihydrobiopterin (BH_2) by dihydrofolate reductase (DHFR), termed as the salvage pathway [17]. However, it is not known whether sex hormones and/or their receptors alter nNOS activity, nitroergic-mediated gastric function, or BH_4 synthesis enzyme expression in an experimental hyperglycemia.

Estrogens mediate their biological actions through their respective nuclear (genomic) and cytoplasmic/membrane (non-genomic, rapid) receptors. Estrogen receptors (ERs) have two classical subtypes, ER α and ER β , expressed from two distinct genes. Several studies demonstrated that both ERs are localized in nerve cells of the gut [18, 19]. E_2 has multiple beneficial actions that include neuroprotection, maintaining glucose homeostasis, and activation of NO synthesis in vascular smooth muscle [20–23]. Although many studies aim to understand how E_2 (non-selective ER agonist) can promote various cellular effects, selective activation of ERs may prove useful in novel treatment options for many diseased conditions. In addition, many studies highlight the fact that selective activation of ER α or ER β may produce similar or opposing effects in different tissues [24, 25].

Interestingly, estrogens are known to be protective against oxidative stress in various cell types, yet the association between hormones and oxidative stress in gastroparesis has been elusive [20, 26]. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), an antioxidant-responsive transcription factor, has emerged as a potent target for alleviating clinical manifestations of diabetes mellitus (DM), in addition to its regulation

of gastric nitrergic function [27–29]. Under oxidative stress, Nrf2 induces transcription of Phase II genes for detoxification and neutralization of reactive oxygen species (ROS) to protect against oxidative damage as manifested in diabetes mellitus. One of the primary protective downstream target of Nrf2 is heme oxygenase 1 (HO-1), which has been shown to be protective to NO synthases in the vasculature [30, 31]. Our previous studies have shown that activation of Nrf2 can regulate antioxidant mechanisms, neuronal nitric oxide (nNOS)-mediated gastric function, and estrogen receptor expression in obesity-induced diabetic gastroparesis [27]. However, the contribution of distinct estrogen receptors on gastric Nrf2, Nrf2-Phase II detoxifying enzymes and nNOS function is unknown in experimental hyperglycemia.

Taken together, the objective of this study is to investigate whether selective activation of gastric estrogen receptors improves gastric Nrf2-nNOS expression in an in-vitro experimental hyperglycemic model. We hypothesize that estrogen and selective ER agonists regulate Nrf2/nNOS-mediated gastric motility and emptying in diabetic females.

2. Methods

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Meharry Medical College (MMC), in accordance with recommendations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Adult female C57BL/6J (n=48, 12-15 weeks, 18.93 ± 0.272 g) mice were purchased from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, ME). All animals were housed in the institutional animal care vivarium under standard conditions (4 mice/cage, 12hr light cycle) and allowed access to food and water ad libitum.

Estrus Cycle Assessment:

To exclude interference from endogenous sex hormones, all mice used in this study were selected during the diestrus stage of the reproductive cycle. The diestrus stage has been characterized by lower sex hormone ($E_2 + P_4$) levels [32, 33]. On the morning of the experimental day, vaginal smears were performed by flushing with ~50 ul of sterile phosphate buffered saline (PBS). The fluid was then carefully placed onto a glass slide and observed for predominant cell types presents under a light microscope at 10x magnification. All mice used for in-vitro studies were healthy and only handled for estrous cycle determination prior to euthanization; therefore we report no adverse events.

Experimental Design and Tissue Culture Experiments:

On the day of experimentation, groups of healthy mice (n=4/group, 11 total groups) were euthanized via CO₂ asphyxiation. Full-thickness stomach and duodenum specimens were immediately isolated and placed in oxygenated Krebs's physiological buffer (pH, 7.4). Gastric neuromuscular strips were randomized and incubated in various conditions for 48 hours and grouped accordingly: (1) normoglycemic (NG; DMEM (5.5 mM glucose)), (2) mannitol (MAN; DMEM + 50 mM mannitol). Two concentrations of hyperglycemia were utilized, (3) 30 mM (DMEM (5.5 mM) + 24.5mM D-glucose) and (4) 50 mM (DMEM (5.5 mM) + 44.5mM D-glucose) glucose. In a separate set of experiments, gastric neuromuscular tissues

were exposed to concentrations of (5) estradiol-17- β (E_2), (6) Progesterone (P_4), (7) 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), or (8) diarylpropionitrile (DPN) in (9) high glucose (30 mM) respectively. All the compounds screened at different concentrations were chosen based on previously published in smooth muscle function [24, 34–36]. Similarly, the effects of gastric sex hormone receptors with (10) ICI 182, 720 (non-selective ER antagonist) and (11) RU-486 (progesterone receptor antagonists) 48 hr were observed in NG conditions. At the end of each incubation, tissue specimens were quickly blotted dry, weighed and snap frozen in dry ice.

Total Nitrite Concentration Estimation:

At the end of 48 hr tissue incubation, all media samples were collected and stored at -80°C . Total nitrite was measured from cultured media via colorimetric assay based on manufacturer's protocol (BioVision, Inc., Milpitas, CA, USA).

Organ Bath Studies:

Electric field stimulation (EFS)-induced NANC relaxation was studied in circular gastric antrum neuromuscular strips, as previously described [8, 27]. The serosa layer was removed; however, the mucosal layers were intact for our studies. Circular gastric antrum neuromuscular strips were mounted between two L-shaped tissue hooks in 5 mL water jacketed organ baths containing Krebs buffer (pH 7.4) at 37°C and continuously bubbled with 95% O_2 , 5% CO_2 (DMT-USA, Inc, Ann Arbor, MI). Tension for each neuromuscular strip was monitored with an isometric force transducer and analyzed by a digital recording system. A passive tension equal to 2 g was applied to each strip during the 1 hr equilibration period through incremental increases (0.5 g, four times, at 15 min intervals). Neuromuscular strips were incubated with atropine, phentolamine, and propranolol (10 μM each) for 30 min to block adrenergic and cholinergic responses. Strips were precontracted with serotonin (5-HT 100 μM) and were exposed to EFS (1ms pulse) for 1 min duration to elicit NANC relaxation. The antrum neuromuscular strips were stimulated at 2 Hz and the resulting changes in response were measured for the baseline nitroergic relaxation. Then the neuromuscular strips were exposed to HG (30 mM) for 90 mins and were measured for nitroergic relaxation. To determine the effect of exogenous ovarian hormones or ER activators on nitroergic function, gastric antrum neuromuscular strips were pre-incubated in the presence of 30mM HG simultaneously with E_2 , P_4 , PPT, or DPN for 90 mins. After incubation, the NANC-mediated relaxation was recorded.

In a separate set of experiments, the effect of ICI 182, 720 (non-selective ER antagonist) incubation on nitroergic relaxation in NG was measured. In each study, relaxation response elicited by low-frequency (2 Hz) stimulus under NANC conditions was predominantly nitroergic in origin. The NO dependence of NANC relaxations was confirmed by preincubation with N-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 30 min; 100 μM , Sigma, St. Louis, MO). At the end of the experiment, each muscle strip was blotted dry with filter paper and weighed. Comparisons between the groups were performed by measuring the area

under the curve (AUC) of the EFS-induced relaxation (AUCR) for 1 min and the baseline for 1 min (AUCB), according to the formula: $(AUCR-AUCB)/\text{weight of the tissue (mg)} = \text{AUC/mg tissue}$.

RT-qPCR

Gastric antrum neuromuscular tissues were harvested from the treatment groups were snap frozen in liquid nitrogen. Total RNA was extracted using TriZol-Reagent (Thermo Fisher Scientific, Waltham, Ma) as described by the manufacturer's protocol (Molecular Research Center, Thermo Fisher, Waltham, Ma). The iScript cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA. One microliter of cDNA was used for each reaction, and the primers represented in Table 1 were used. RT-quantitative PCR (RT-qPCR) amplification was performed using the SYBR Green (Bio-Rad, Hercules, Ca) method. Cycling conditions were 95°C for 3 min, followed by 45 cycles of 95°C for 30 s and 55°C for 1 min. Relative amounts of mRNA were normalized to β -actin and threshold cycle (CT) numbers were calculated (i.e., $2^{-\Delta\Delta CT}$, the Ct method), according to the manufacturer's instructions (Bio-Rad). All studies were performed in the MMC Molecular Core Laboratory.

Western Blot Analysis

Full-thickness gastric neuromuscular specimens were homogenized, and protein were estimated in each of the lysates. Equal concentrations of protein lysates (40 μg) were separated on a 6% and 12% SDS polyacrylamide gel then transferred to nitrocellulose membrane. Each membrane were blocked with 5% dried non-fat milk for 1 hr, then incubated with primary polyclonal antibody [ER α , (1:500), ER β (1:500), DHFR (1:500), Nrf2 (1:1000), and HO-1 (1:1000) purchased from (Santa Cruz Biotechnology, Santa Cruz, Ca)] overnight, respectively. Expression of nNOS α was detected using rabbit polyclonal antibody at 1:1000 (Abcam, Cambridge, Ma). The membranes were washed 3 times for 10 min each in 0.01% TBS-Tween, then incubated in horseradish peroxidase-conjugated secondary antibody (1:1000) for 1 hour at room temperature. The blots were visualized with ECL Western Blotting Detection Reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and ChemiDoc Touch Imaging System (BioRad, Hercules, CA). The reactive bands were analyzed quantitatively by optical densitometry. The blots were stripped and re-probed to measure protein expression. Blots were re-probed with β -actin polyclonal antibodies (1:5000) (Sigma, St. Louis, MO) to enable normalization of signals between samples. Band intensities from Western blot images were analyzed using Image Lab Software (BioRad Hercules, Ca)

Statistical Analysis

Data were presented as the mean \pm standard error (SE). For this study, we employed a method of sample size calculation based on the "resource equation" method, as previously detailed[37]. 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

Effect of Estrogen Receptor Antagonist, ICI 182,780 and Progesterone Receptor Antagonists, RU-486, on Gastric Nitroergic Relaxation in Normoglycemic Conditions.

Gastric neuromuscular specimens exposed to ICI 182,780 (1 nM, 10 nM) significantly ($p < 0.05$) reduced nitroergic relaxation. As shown in Fig. 1, this effect was measurable at 10 nM concentration as early as 60 mins, persisting to 120 mins (ICI 10 nM vs. baseline). RU-486 (100 μ M), a common progesterone antagonist did not alter nitroergic relaxation in these tissues during the 2-hour protocol.

Effects of Estrogen and Progesterone Receptor Antagonist on Gastric nNOS, Nrf2 and BH₄ biosynthesis enzymes (GCH-1/DHFR) Expression

To explore the role of sex hormone receptors on gastric Nrf2/nNOSa expression, gastric neuromuscular tissues were incubated in NG conditions for 48 hours either with common ER antagonist, ICI 182,780 or PR antagonist, RU-486. Alterations in mRNA and protein expression were presented in Figure 2. Non-selective blockade of gastric ERs with ICI 182,780 reduces gastric (Fig 2A) nNOSa, (Fig 2B) Nrf2, (Fig 2C), DHFR and (Fig 2E-F) estrogen receptor (a/b) mRNA expression. No measurable effect of RU-486, the progesterone receptor antagonist, was observed when compared to DMEM (NG) control group. In addition, no changes were observed with GCH-1 with either group. Similarly, protein expression of (Fig 3A) nNOSa, (Fig 3B) ERa, and HO-1 were significantly reduced in the presence of sex hormone antagonists, ICI 182,780 and RU-486. As shown in Fig 3C, Nrf2, expression was reduced with ER antagonism. Interestingly, ERb expression (Fig 3D) was significantly ($P < 0.05$) increased in the presence of ICI 182,780 when compared to the control group. At lower concentrations of RU-486, no measurable change was observed in (Fig 3E) HO-1 expression.

Sex Hormones and Selective ER Activation Restored Hyperglycemia-induced Reduction of Nitroergic Relaxation in Gastric Antrum Specimens

Since ER antagonists reduced nitroergic relaxation in NG conditions, we next investigated whether E₂ and/or ERs restore impaired (Fig 4A-D) nitroergic relaxation in gastric antrum specimens exposed to in-vitro HG. Circular gastric antrum neuromuscular strips were mounted and incubated in hyperglycemia in the absence or presence of 17 β -estradiol (E₂) (Fig. 4A), progesterone (Fig. 4B), or selective ER agonists, PPT (Fig. 4C) or DPN (Fig. 4D). Nitroergic relaxation was significantly ($p < 0.05$) impaired in hyperglycemia. E₂, at both concentrations (10 nM, 100 nM), restored nitroergic function. While activation of ERa with PPT effectively attenuated hyperglycemia-induced nitroergic dysfunction in neuromuscular strips at 100 nM concentration only (4C), ERb activation with DPN rescued nitroergic relaxation at both concentrations (1 μ M, and 10 μ M) (4D). Progesterone did not alter nitroergic relaxation in hyperglycemia. To confirm the relaxation response was nitroergic in origin, neuromuscular specimens incubated with L-NAME (nNOS blocker) display attenuation of gastric relaxation.

Sex Hormones and Selective ER Agonists Restore Nrf2/ nNOSa Expression and Total Nitrite Production in Hyperglycemic Conditions

Our data demonstrate a significant ($p < 0.05$) reduction in (Fig 5A) nNOS α , (Fig 5B) Nrf2, (Fig.5C) GCH-1, (Fig 5D) DHFR and (Fig 5 E-F) estrogen receptor (a/b) mRNA expression in hyperglycemic conditions. To assess the effect of hyperglycemia in gastric neuromuscular specimens, gastric neuromuscular specimens were incubated in the presence of high glucose (30 or 50mM) for 48 hours (Fig 6 A-C). The effects of elevated glucose levels on cellular osmolarity is reported [38, 39]. Thus, an isomer of glucose, mannitol was used as an osmotic control group in these studies [40]. Western blot analysis revealed no substantial change in the relative expression of (Fig 6A) nNOS α , (Fig 6B) Nrf2, or (Fig 6C) ER α within the mannitol treated groups. Gastric tissues exposed to experimental hyperglycemia (30 mM or 50 mM), significantly ($p < 0.05$) reduced nNOS α , Nrf2, and ER α levels. However, no differences were observed between hyperglycemic groups.

To assess whether sex hormones or selective ER activation altered Nrf2/nNOS protein expression, gastric neuromuscular tissues were incubated in hyperglycemia in the absence or presence of selective ER agonists, PPT or DPN. As seen in Fig 6 D-F, selective ER α / β activation (PPT/DPN) restored (Fig 6D) nNOS α , (Fig 6E) ER α , (Fig 6F) Nrf2 protein expression in hyperglycemia. Moreover, (Fig 6G) ER β expression was significantly upregulated in hyperglycemia, but restored by PPT. HG altered HO-1 levels, changes were noted in the presence of PPT and DPN.

Since duodenal motility contributes to overall gastric motility and emptying function, we next investigated whether Nrf2/nNOS α expression is also affected in HG conditions. As shown in Figure 7, in-vitro hyperglycemia significantly ($p < 0.05$) reduced (Fig 7A) nNOS α and (Fig 7B) Nrf2 expression in duodenum specimens. As shown in Fig 8A, sex hormone antagonists, ICI 182,780 and RU-486 decreased total nitrite production in-vitro. Similarly, total nitrite levels, a measure of NO production were significantly reduced in hyperglycemia. (Fig. 8B) PPT was effective at the higher concentration (100 nM) only. In contrast, DPN effectively attenuated reduced nitrite levels in hyperglycemic conditions at both concentrations (1 μ M, and 10 μ M).

4. Discussion

Gender bias in gastric emptying is well-documented in both health and gastroparesis; however, the role of endogenous sex hormones in regulating gastric motility remains unclear. Women during their reproductive ages, tend to be disproportionately affected by gastroparesis because their stomach motility is slower to begin with, likely due to elevated levels of sex steroid hormones and nitric oxide. The pathogenesis of diabetes-associated motility disorders are multifactorial, though much can be attributed to abnormalities in nitric oxide/nNOS expression, enzyme activity and oxidative stress [41]. Our results show that in-vitro hyperglycemia reduces 1) gastric and duodenal Nrf2, nNOS α and ER alpha protein expression, 2) supplementation of estrogen and/or estrogen receptors restores Nrf2, nNOS α , total nitrite and nitrenergic relaxation in hyperglycemic conditions. In addition, pre-incubation with ER antagonists inhibit Nrf2 and nNOS α protein expression and nitrenergic relaxation. Collectively, the above studies suggest that estrogens regulate gastric motility via stimulating Nrf2/nNOS α signaling mechanism(s).

Estrogen receptor signaling is complex, but it is well understood to primarily mediate many of their biological effects via genomic regulation. Most of the actions of estrogens appear to be exerted via two estrogen receptor (ER) subtypes, denoted ERa and ERb, intracellular proteins that are members of a large superfamily of proteins that function as ligand-activated transcription factors [22]. Although ER subtype surface membrane receptors exist, few studies have implicated these targets in the rapid vasodilator effects of E₂ [23]. Moreover, several lines of evidence suggest that both ERa and ERb proteins are expressed in enteric neurons within the nucleus and cytoplasm [19]. Co-expression of ERa and ERb in enteric neurons indicates that estrogenic effects could also be mediated through neurogenic reflexes [16, 18]. Therefore, our study sought to understand the genomic changes associated with selective ER activation in gastric neuromuscular specimens.

To test whether sex hormone receptors contribute to gastric nitrgic function, we have first investigated the effects of non-selective antagonists: ICI 182, 780 (ER) and RU-486 (PR) in ex-vivo normoglycemic conditions. Our studies demonstrate that blockade of estrogen receptors, but not progesterone receptors, by antagonists showed a reduction in nitrgic relaxation in gastric neuromuscular strips; implicating ER signaling/effects. Estrogens have been shown to mediate both, rapid and genomic, effects. Genomic effects of steroid hormones have been shown to occur in as early as 2 hours [42]. The results from our study revealed that mRNA and protein expression for Nrf2/nNOSa are altered by inhibition with ER or PR antagonists which clearly shows that total nitrite production is also altered in the presence of ICI and RU-486. The above data suggests that ERs could play a vital role in gastric motility function through regulating Nrf2/nNOSa expression and nitrgic relaxation.

Furthermore, several cofactors have been shown to be important for nNOSa activity, including BH₄. The level of BH₄ is tightly regulated by both *de novo* and salvage pathways. GCH-1 is a rate limiting enzyme and regulates BH₄ levels via the *de novo* pathway, while DHFR reduces oxidized (inactive) BH₂ and B to active BH₄ via the salvage pathway. Previous studies from our laboratory suggest E₂ deficiency reduced expression of GCH-1 and DHFR levels in female follitropin receptor knockout (FORKO) gastric neuromuscular tissue [8]. In addition, reduced levels of E₂ may augment impairment of BH₄-nNOSa function and elevate oxidative stress, thus promoting gastroparesis in women. Our studies show that inhibition of ER, but not PR, reduces DHFR, but not GCH-1 suggesting that ERs perhaps synthesizing BH₄ via the salvage pathway.

Our current studies demonstrate that in-vitro exposure to HG significantly reduced gastric protein expression of both ERa and ERb, Nrf2/nNOSa, total nitrite and nitrgic relaxation. Although the osmolarity of the culture medium was not assessed, the effects of elevated glucose levels on cellular osmolarity is well reported. Exposure to high glucose concentrations in vitro is often used to understand the cellular modifications occurring in diabetes[39]. Furthermore, recent studies have documented that hyperosmolarity, as occurring in diabetic hyperglycemia, may represent important regulatory elements influencing cell fate and viability, both in physiological and pathological conditions. These studies further report using glucose concentrations between 24mM – 75 mM with incubation times up to 72 hr are

sufficient to mimic the diabetic oxidative stress response in different cellular types (human gingival fibroblasts and erythrocytes) [39, 43]. Furthermore, exposure to a prolonged severe hyperglycemic (>30mM) load is correlated with increased susceptibility to cellular damage and severe inhibitory effects on nNOS/Nrf2. However, short term incubation (<48 hr) display little effect on cell viability while maintaining HG insult. In our study, we examined the gastric neuromuscular response to increasing concentrations of hyperglycemia (DMEM(5.5mM), 30 mM and 50 mM glucose) in the culture media for 48 hr. (Fig 6 A-C). Although we observed no significant differences between nNOS, ER, and nrf2 protein expression in 30 mM and 50 mM glucose media, the remaining HG experiments (incubation with hormones/ER agonists/antagonist and organ bath studies) employed a lower (30 mM) glucose concentration in the incubation media.

In addition, our studies show that nonselective (E_2), and selective activation of ER α by PPT or ER β by DPN restored gastric Nrf2/nNOS α expression, total nitrite and nitrenergic relaxation in vitro exposed to HG. After 90 min incubation, we observe differences in the efficacy of the selective ER agonists at various doses; though each ER agonists significantly enhanced nitrenergic relaxation in gastric neuromuscular specimens exposed to in-vitro hyperglycemia. This is in line with Al-Shboul et al., reporting that estrogen-induced relaxation was greater in female gastric smooth muscle cells (GSMC) compared with that in male [44]. Our current studies further asserted that ER α agonist, PPT and the ER β agonist, DPN induced relaxation to a greater extent than PPT, although this result was not statistically significant. These differences may be due to variations in receptor subtype expression in GSMCs vs enteric neuronal cells. All of our data were generated from gastric full-thickness specimens that has several cellular subtypes including smooth muscle and enteric neuronal cells. Furthermore, as shown in Fig. 2., selective activation with PPT restored Nrf2, nNOS α , ER α , dihydrofolate reductase (DHFR) mRNA expression; selective ER β activation (DPN) restored nNOS β , Nrf2, and ER β mRNA expression. Collectively, the above studies suggest ER activators regulate nitrenergic relaxation by restoring gastric Nrf2/BH $_4$ synthesis in an experimental hyperglycemic condition.

Estrogen receptors (α/β) are encoded by separate genes and exhibit distinct tissue distributions[18, 23, 45–47]. ER α is well known to be majorly expressed in the uterus, liver, kidney and heart. Whereas, ER β expression is primarily found in the ovary, lung, gastrointestinal tract, bladder, and central nervous systems. Much of the evidence describes spatially-specific actions in which ERs may possess converging or diverging pathways leading to cellular responses[27, 42]. It has been demonstrated in bone that ER β can stimulate some of the same genes as does ER α , whereas ER β almost always reduced the magnitude of gene stimulation by ER α when both receptors were present. It has also been reported through the use of subtype-selective ligands that ER β modulation of ER α activity appears to be response specific. At experimental doses, PPT displays 410-fold selectivity for ER α over ER β , whereas DPN displays a 70-fold selectivity for ER β [34, 48]. However, Tamir et al. reported that oxidative stress, a well-known consequence of diabetes, differentially regulates the expression of ER α and ER β in various cells [22]. Furthermore, although not studied in this context, numerous mRNA splice variants exist for both ERs in diseased and healthy tissues. These splice variants are speculated to potentially alter full-length (active) protein

expression and activity in rodent studies, potentially facilitating compensatory signaling mechanism[49–51]. Our findings demonstrate that ER beta is increased in hyperglycemia. We anticipate that PPT activation of ER alpha may restore other mechanisms (i.e inflammation) to prevent the upregulation of ER beta in HG. Future studies are needed to further understand the complexities of sex hormone receptor signaling. Furthermore, the expression of ERs, DHFR, Nrf2, and HO-1 could be from mucosal, muscle and neuronal layers. We did not conduct the cellular localization studies of the target proteins in the current study. Of note, several studies have demonstrated that nNOS and ERs are primarily localized within enteric neuronal cells of the gut [16, 52]. Furthermore, regionally specific co-localizations of nNOS and ER α have also been reported, suggesting potential interaction in this system within the neurons. Reports indicate that enteric neurons innervate throughout the layers of the stomach[12, 18, 19]. Therefore, we speculate that despite of localization of these specific markers in various cell types (mucosal -> neurons), may interact with via autocrine and/or paracrine fashion, to thus regulate nNOS-mediated motility of the stomach.

Moreover, our previous studies demonstrate that loss of Nrf2 reduced nitrenergic relaxation and delayed gastric emptying in female mice [27, 28]. In-vivo activation of Nrf2 has been shown to regulate gastric nNOSa function and ERs in a high-fat diet fed obesity Type II DM model [27]. Much of the work delineating the interactions between Nrf2 and ovarian hormone receptors is limited to breast cancer models [53, 54]. Estrogen (E₂) increases Nrf2 activity in MCF7 breast cancer cells through activation of the PI3K/GSK3 β non-genomic pathway [53]. Similarly, E₂ is known to regulate, to a lesser extent, antioxidant response element (ARE)-responsive genes through Nrf2 and co-activators within the promoter region of these genes [53, 55, 56]. Our study sought to provide evidence for genomic changes in Nrf2 and nNOS in response to selective ER activation.

In addition, Nrf2 is widely known to bind to a host of Phase II detoxifying enzymes to rid the cell from oxidative stressors. Most importantly, heme oxygenase 1 (HO-1) is a protective marker controlled by Nrf2-ARE. In mouse models of diabetes, increased expression of antioxidants such as HO-1 protected ICC from oxidative stress and reversed diabetic gastroparesis [31]. Here, we provide evidence of a reduced expression of HO-1, that was restored by selective ER activators, suggesting ER alpha and beta can increase HO-1 expression. These findings suggest co-activation with nuclear ERs can facilitate HO-1 gene expression.

Furthermore, motor abnormalities associated with gastroparesis syndrome may not be limited to the smooth muscle function. Diabetic gastroparesis comprises a decrease in fundic and antral motor activity, a reduction or a lack of the inter-digestive migrating motor complex, gastric dysrhythmias, and pylorospasms [13, 57]. As reported earlier, NO donors were ineffective in relieving gastroparesis symptoms in humans suggesting that stomach motility is not solely regulated by smooth muscle [58]. Numerous studies demonstrated that gut motility is regulated by enteric neuronal system including nNOS. Therefore, we suggest that although NO donors have an effect on relaxing smooth muscle in general, it may not relieve gastroparesis symptoms. In our study, hyperglycemia significantly reduced the expression of Nrf2 and nNOSa in gastric antrum and duodenum specimens. We further observed

attenuation of Nrf2 and nNOSa protein expression with sex hormone (primarily E₂) supplementation. Earlier studies in duodenum report the number of nNOS nerve cell bodies per ganglia was increased in type II DM rodent models of gastroparesis; however, the density index of nNOS varicosities was reduced [14, 59]. These studies further suggested that nitrergic neurons might be protected from hyperglycemia-related oxidative stress better in the duodenum. Similarly, the impairment of nNOS pathways in streptozotocin (STZ)-induced diabetic rats, the nitrergic myenteric neurons did not diminish in the duodenum, unlike the other gut segments. [31]. In the diabetic duodenum, besides a decreasing number of nNOS neurons, the number of colocalized myenteric neurons did not alter significantly. Our studies so far align with a decreased expression of nNOS in the duodenum, however sex hormones may improve complications associated with this depletion of NO. It should be noted that Celtek, and others, suggest that nNOS expression is reversibly decreased (point of return) in the nitrergic axons while unaffected in the cell bodies in the early stages of diabetes in male rodents[60]. The same studies further suggest that nNOS neurons are reduced in the long-term diabetes (point of no return). Since hormones and their receptor agonists were able to restore nNOS protein expression, we suggest that our experimental HG model is related to the early stages, but not long-term diabetic conditions in which we would expect a loss of nNOS neurons.

5. Conclusions

Taken together, these findings imply that direct regulation of multiple cellular key molecules by estrogens may contribute to the modulation of gastric functions that have been recognized during hyperglycemic conditions. In the current study, we demonstrate the relevance of sex hormones and gastric estrogen receptors in mediating nitrergic relaxation and Nrf2/nNOS expression- both critical to gastrointestinal motility. Experiments are underway to investigate the in-vivo effects of hyperglycemia and sex hormones/receptor modulators on Nrf2- phase II antioxidant enzyme expression in stomach and duodenum. Linking symptoms to physiology and to cellular changes are important steps in determining the key targets for therapy design. A comprehensive identification of signaling pathways involving sex hormone receptors and Nrf2 activators on nitrergic function in diabetic rodent model may be beneficial targets to alleviate symptoms associated with diabetic gastroparesis in females. Drugs that can selectively modulate the activity of either ER α or ER β in their interactions with target genes may represent a promising frontier in gastric dysmotility coadjuvant therapy. Taken together, these findings provide insight into gender-related differences observed in gastric motility via experimental hyperglycemia.

Abbreviations

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), neuronal Nitric Oxide Synthase (nNOS), hyperglycemia (HG), normoglycemic (NG), estrogen receptor (ER), progesterone receptor (PR), Gastrointestinal (GI), diabetes mellitus (T1/T2DM), gastric emptying (GE), dopamine-2 (D2), nitric oxide (NO), interstitial cells of Cajal (ICCs), non-adrenergic, non-cholinergic (NANC), 17 β -estradiol (E₂), tetrahydrobiopterin (BH₄), guanosine triphosphate (GTP), GTP cyclohydrolase I (GCH-1), dihydrobiopterin (BH₂), dihydrofolate

reductase (DHFR), reactive oxygen species (ROS), Institutional Animal Care and Use Committee (IACUC), Meharry Medical College (MMC), National Institutes of Health (NIH), progesterone (P₄), phosphate buffered saline (PBS), mannitol (MAN), 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), diarylpropionitrile (DPN), Dulbecco's modified eagles media (DMEM), Electric field stimulation (EFS), serotonin (5-HT), N-Nitro-L-arginine methyl ester hydrochloride (L-NAME), area under curve (AUC), Real-Time quantitative Polymerase Chain Reaction (RT-qPCR), threshold cycle (CT), Analysis of Variance (ANOVA), follitropin receptor knockout (FORKO), gastric smooth muscle cells (GSMC), antioxidant response element (ARE), heme oxygenase 1 (HO-1), streptozotocin (STZ).

Declarations

Ethical Approval and Consent to Participate: All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Meharry Medical College (MMC), in accordance with recommendations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing Interests: The authors declare no competing interests.

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Author Contributions: PG, filed a patent application for the use of BH₄ (tetrahydrobiopterin) in gastroparesis subjects through University of Texas Medical Branch at Galveston (UTMB) Texas, USA. PG conceived and designed research; JS and CS conducted all experiments & acquired and analyzed the data; PG, JS, and CS interpreted results of experiments; JS drafted the manuscript; CS edited manuscript; JS, CS, and PG edited & revised manuscript and have read and approved the final version of manuscript.

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Table

Table 1. List of Primers used for quantitative RT-PCR assays

Gene	Forward	Reverse
ER α	5'- CCTCCCGCCTTCTACAGGT-3'	5'- CACACGGCACAGTAGCGAG-3'
ER β	5'-CTGTGATGAACTACAGTGTTCCC-3'	5'-CACATTTGGGCTTGCAGTCTG-3'
nNOS α	5'-GGTGGAGATTAACATTGCTGTCCTA-3'	5'-TTCTCCATGTGTTTGATGAAGGACT-3'
Nrf2	5'-TCTCCTCGCTGGAAAAAGAA-3'	5'-TAAAGCACAGCCAGCACATT-3'
DHFR	5'-TCGACCATTGAACTGCATCGTCGCC-3'	5'-GGAATGGAGAACCAGGTTTTCTACC-3'
GCH-1	5'- GAGCATCACCTTGTTCCATTTG -3'	5'- GCCAAGTTTACTGAGACCAAGGA -3'
β -Actin	5'-TGGAATCCTGTGGCATCCATGAAAC-3	5'-TAAAACGCAGCTCAGTAACAGTCCG-3'

Figures

Fig. 1

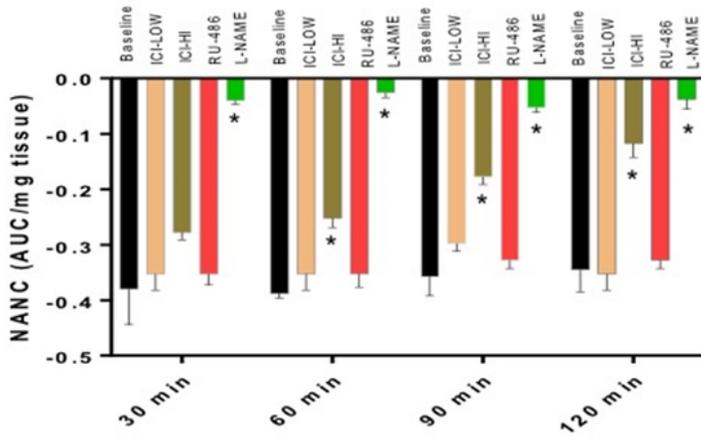


Figure 1

Effect of sex hormone receptor antagonist (ICI 182,780 and RU-486) on nitergic relaxation during 2-hour period. EFS (2 Hz) was used to elicit nNOS-mediated nitergic relaxation from gastric antrum strips every 30 minutes during a 2-hour period. Bar graphs showed changes in nitergic relaxation with various treatments and expressed as area under the curve calculated per milligram tissue weight (AUC/mg

tissue). Data were analyzed using one-way ANOVA by using GraphPad prism software. Data are means \pm SEM (n=4). * P<0.05 compared with normal glycemia (NG) groups.

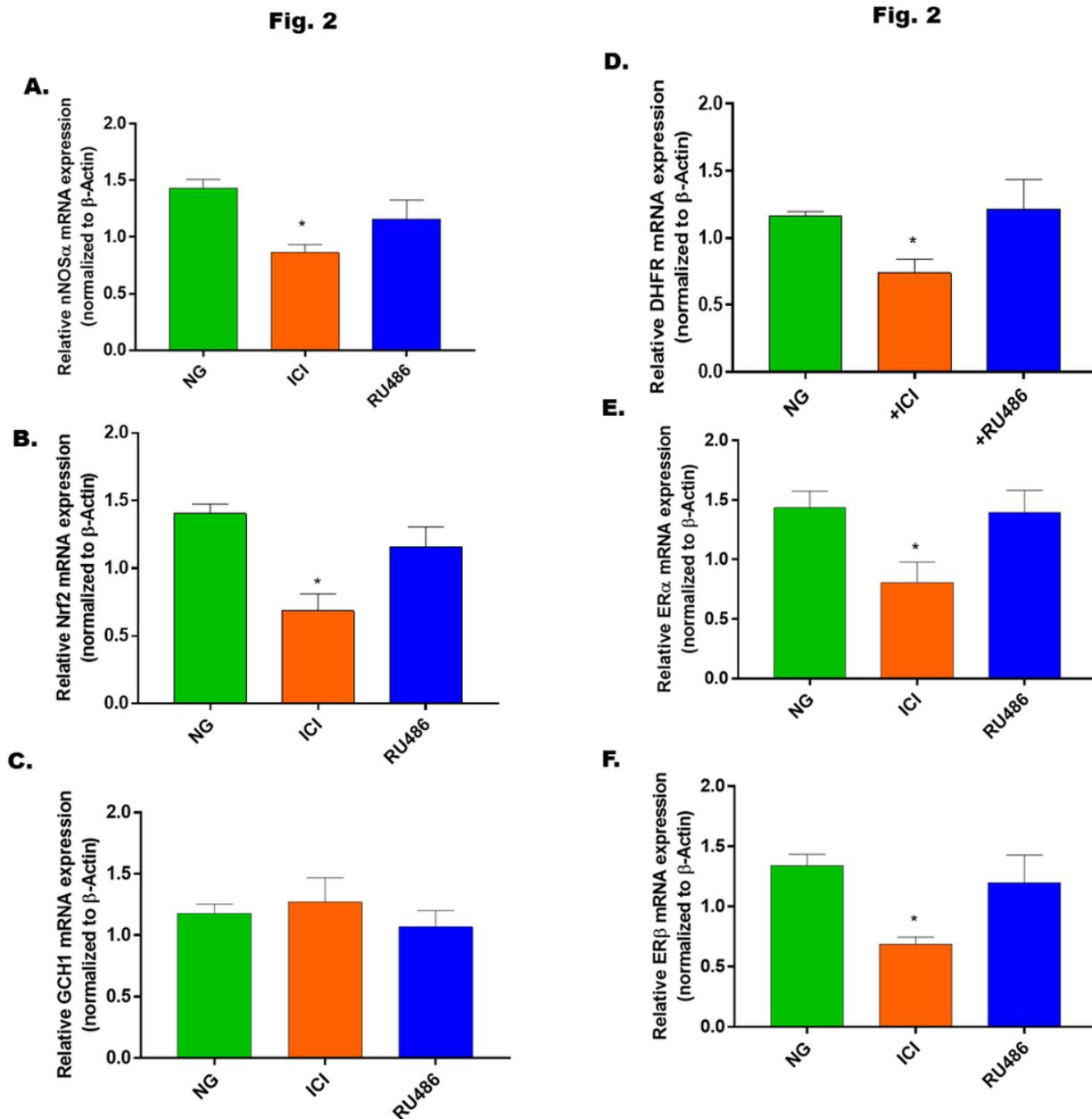


Figure 2

Effect of sex hormone receptor antagonist (ER; ICI 182,780 and PR; RU-486) on NOS α , Nrf2, ER and BH4 synthesis enzyme mRNA expression. Gastric neuromuscular tissues incubated with sex hormone receptor antagonists for 48 hours (2A) nNOS α , (2B) Nrf2, (2C) GCH-1, (2D) DHFR, (2E&F) ER α , ER β mRNA expression were measured via RT-qPCR. Data were normalized with housekeeping gene (β -actin). Bar graphs showed a ratio of target gene or protein with β -actin. Data were analyzed using one-way ANOVA

by using GraphPad prism software. Data are means \pm SEM (n=4). * P<0.05 compared with normal glycemia (NG) groups.

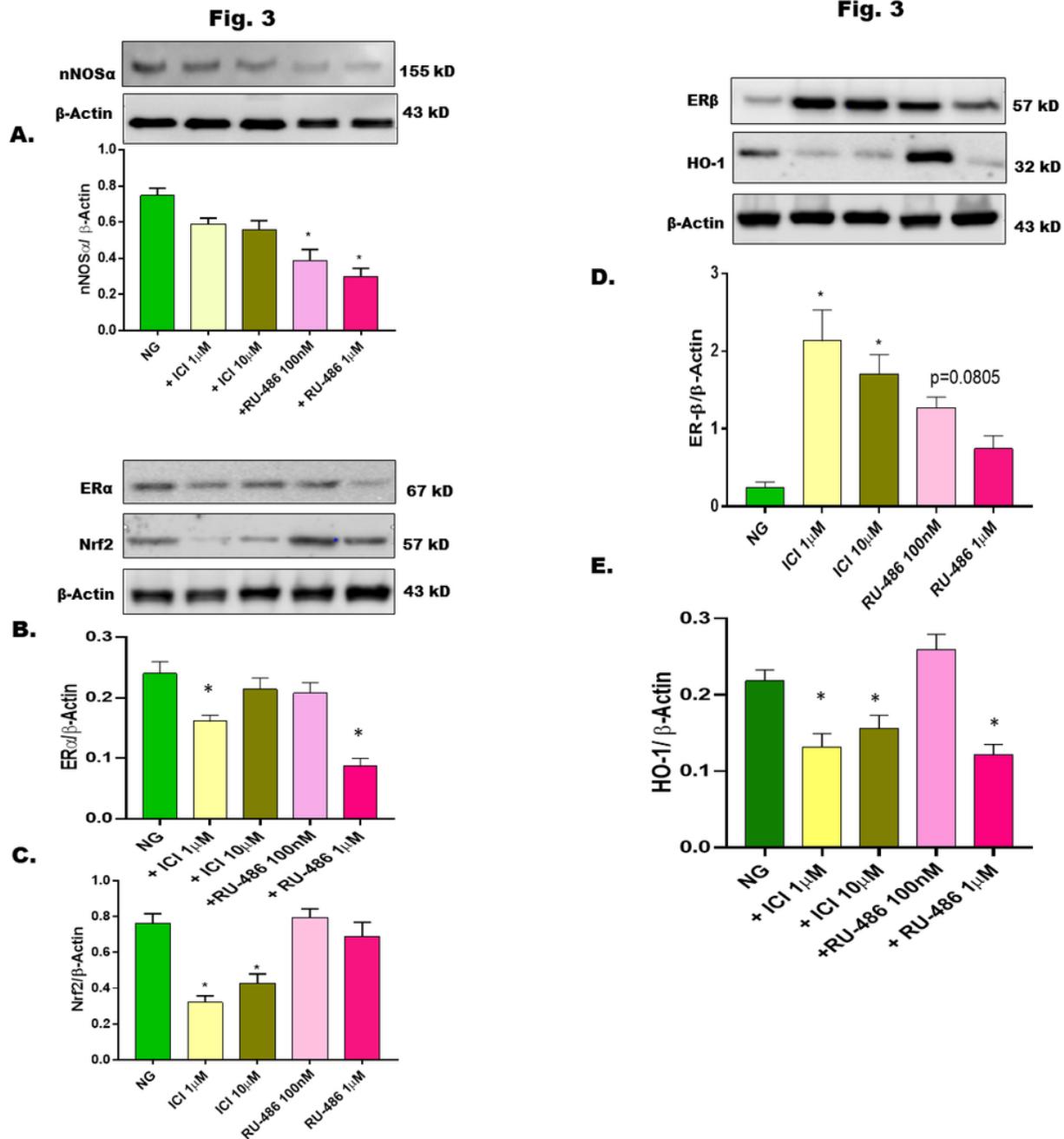
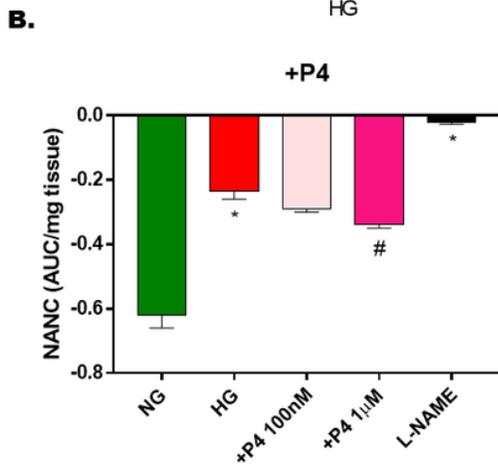
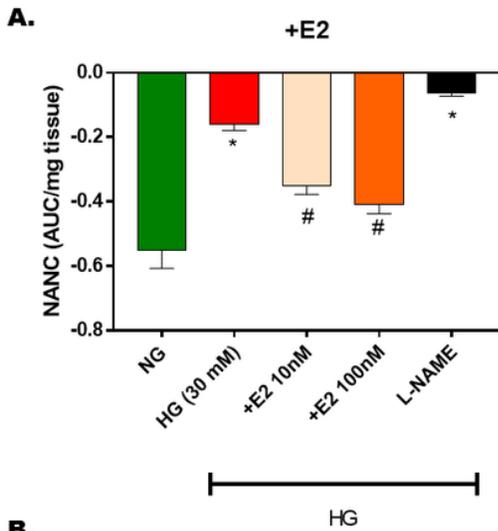
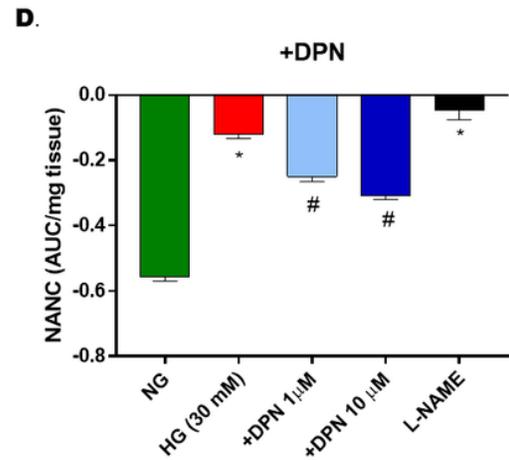
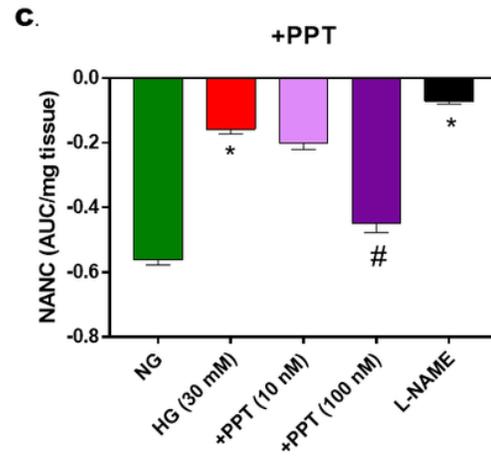


Figure 3

Effect of sex hormone receptor antagonist (ER; ICI 182,780 and PR; RU-486) Nrf2/nNOS expression. Western blot analysis of relative protein expression of (3A) nNOS, (3B) ER, (3C) Nrf2, (3D) ER, (3E) HO-1 are shown. Stripped blots were re-probed with β -actin. Data were normalized with housekeeping 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 GraphPad prism software. Data are means \pm SEM (n=4). * P<0.05 compared with normal glycemia (NG) groups.

Fig. 4**Fig. 4****Figure 4**

Effect of sex hormones and selective ER agonists (ER α ; PPT, ER β ; DPN) on nNOS-mediated nitrgic relaxation in an in-vitro hyperglycemia model. (4A-D) Neuromuscular strips were stimulated via EFS (2Hz) after 90-minute hyperglycemia incubation. Sex hormones (E2 or P4) or ER-selective agonists (PPT, DPN) were pre-incubated for 90 mins in hyperglycemia prior to second stimulation. L-NAME, nNOS blocker, was used to confirm nitrgic response. Bar graphs showed a ratio (AUC/mg tissue). Data were analyzed using one-way ANOVA by using GraphPad prism software. Data are means \pm SEM (n=4). * P<0.05 compared with NG groups. # P<0.05 compared with HG groups.

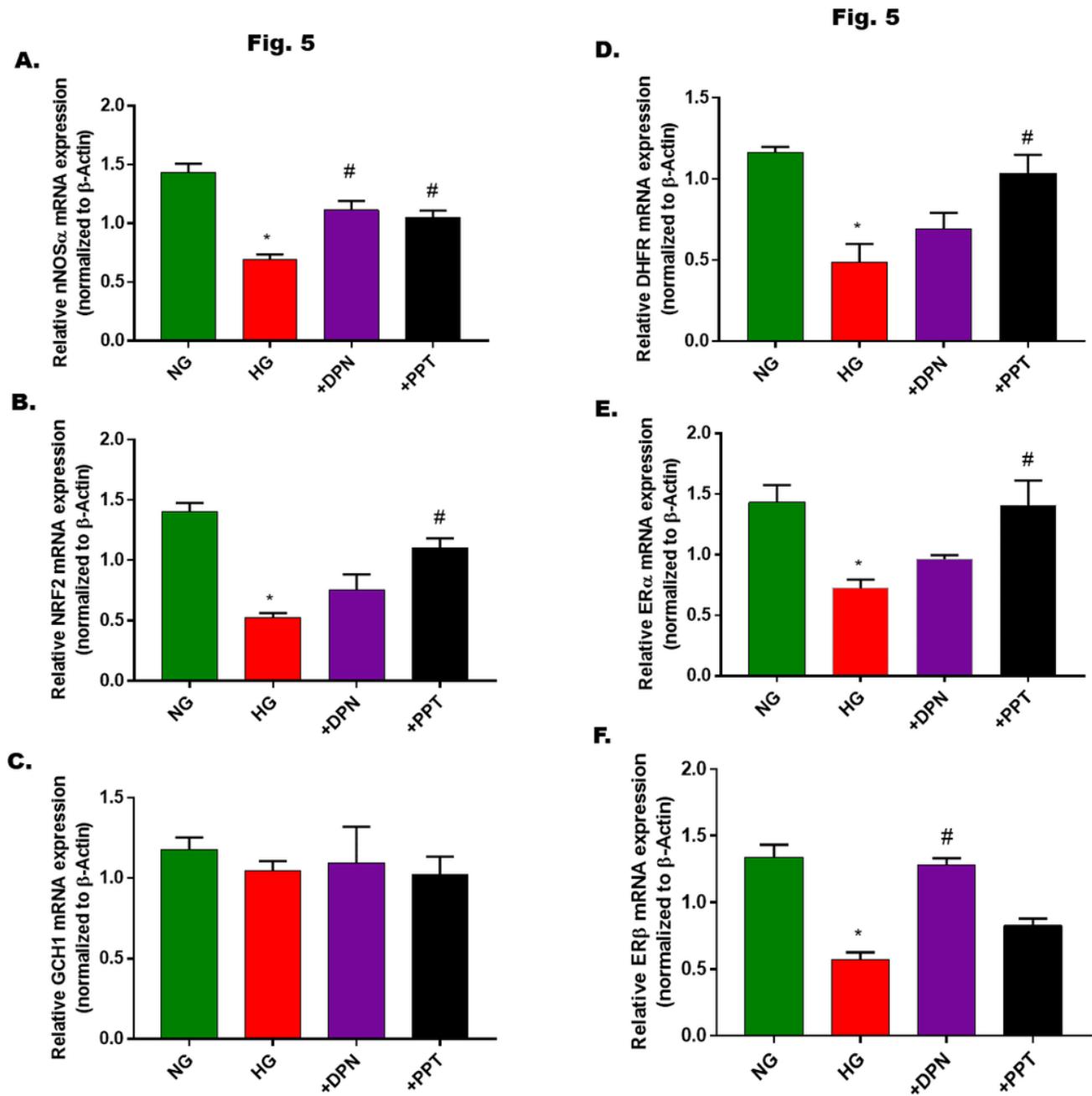


Figure 5

Effect of sex hormones and selective ER agonists (PPT, DPN) on Nrf2/nNOS, ERs, and BH4 synthesis enzyme mRNA expression in an in-vitro hyperglycemia model. (A-D) nNOS-mediated nitrenergic relaxation in hyperglycemia (E-L) nNOS, Nrf2, ERs and BH4 synthesis enzyme expression. Gastric neuromuscular tissues incubated with selective ER-agonists for 48 hours. (5A) nNOS α , (5B) Nrf2, (5C) GCH-1, (5D) DHFR, (5E) ER α , and (5F) ER β mRNA expression were measured via RT-qPCR. Data were normalized with housekeeping gene (β -actin). Bar graphs showed a ratio of target gene or protein with β -actin. Data were

analyzed using one-way ANOVA by using GraphPad prism software. Data are means \pm SEM (n=4). * P<0.05 compared with normal glycemia (NG) groups.

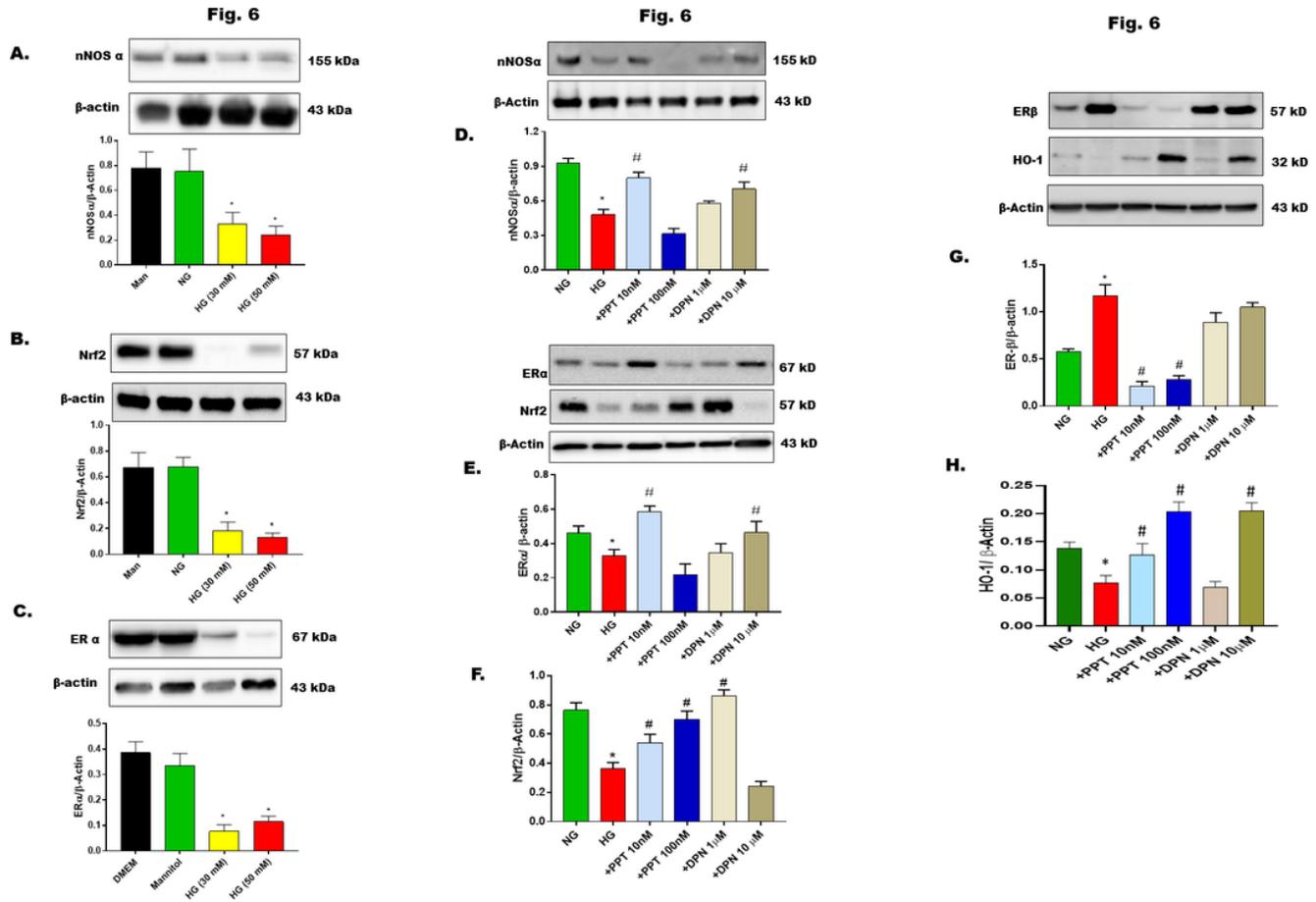


Figure 6

Effect of sex hormones and selective ER agonists (PPT, DPN) on Nrf2/nNOS, ERs, and HO-1 protein expression in 48 hour in-vitro tissue culture. (A-C) The effect of 48 hour in-vitro hyperglycemia was measured on nNOS, Nrf2, ER expression. In a separate set of experiments, the effect of PPT and DPN on (D-H) protein expression was assessed. 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 GraphPad prism software. Data are means \pm SEM (n=4). * P<0.05 compared with NG groups. # P<0.05 compared with HG groups.

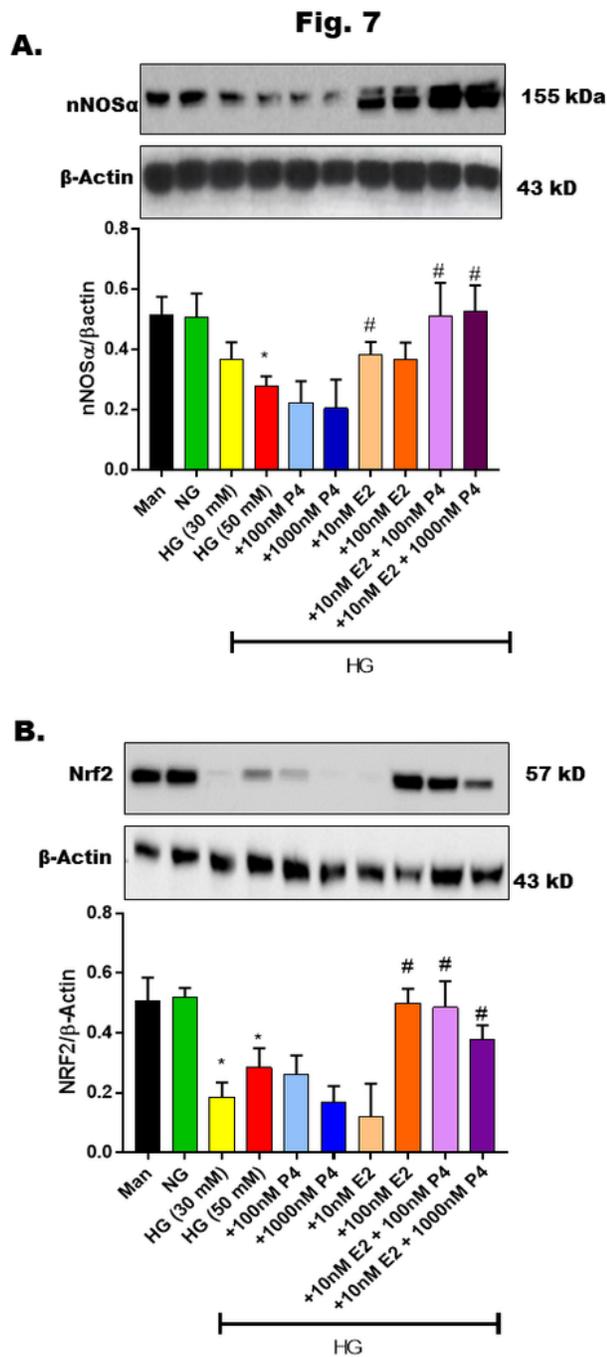


Figure 7

Effect of sex hormones on Nrf2/nNOS expression in duodenum specimens. (A) nNOS expression in hyperglycemia, (B) Nrf2 expression in 48 hour in-vitro experiments. 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 GraphPad prism

software. Data are means \pm SEM (n=4). * P<0.05 compared with NG groups. # P<0.05 compared with HG groups.

Fig. 8

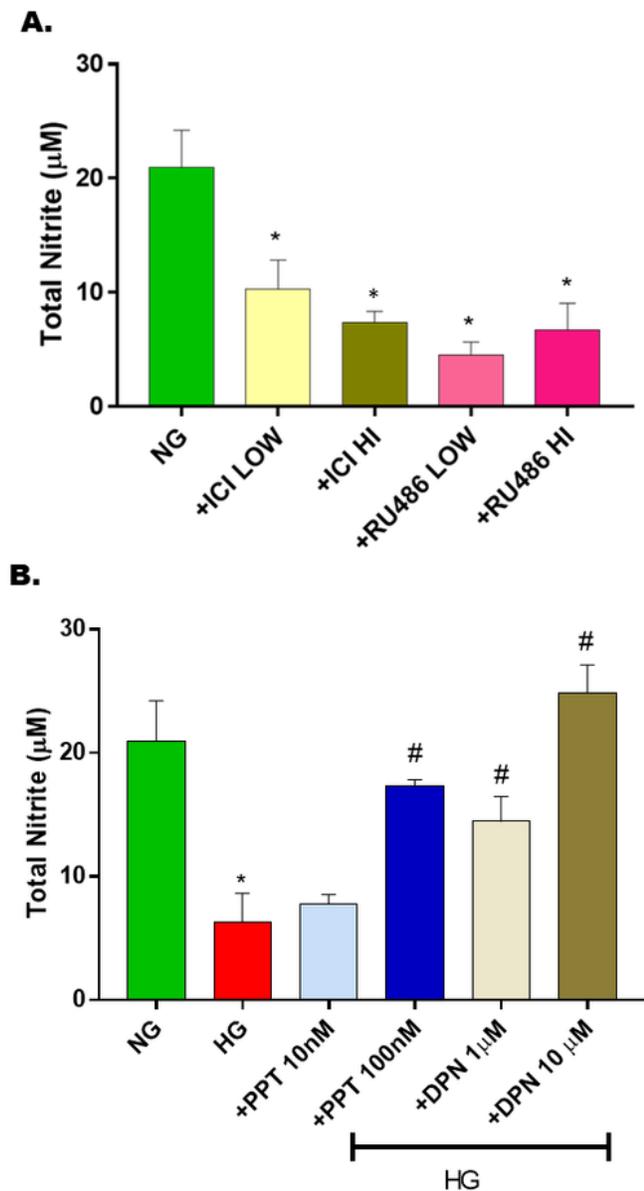


Figure 8

Effect of sex hormone antagonists (ICI 182,780 and selective ER agonists (PPT, DPN) on total nitrite production during 48 hour incubation period from (A) normoglycemia (NG) or (B) hyperglycemia cultured

media. Data were analyzed using one-way ANOVA by using GraphPad prism software. Data are mean \pm SEM (n=4). * P<0.05 compared with NG groups. # P<0.05 compared with HG groups.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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