Diabetes induces sex-dependent changes in neuronal nitric oxide synthase dimerization and function in the rat gastric antrum

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Gangula PRR, Maner WL, Micci M-A, Garfield RE, Pasricha PJ. Diabetes induces sex-dependent changes in neuronal nitric oxide synthase dimerization and function in the rat gastric antrum. Am J Physiol Gastrointest Liver Physiol 292: G725–G733, 2007; doi:10.1152/ajpgi.00406.2006.—Diabetic gastroparesis is a disorder that predominantly affects women. However, the biological basis of this sex bias remains completely unknown. In this study we tested the hypothesis that a component of this effect may be mediated by the nitricergic inhibitory system of the enteric nervous system. Age-matched male and female Sprague-Dawley rats were studied 8 or 12 wk after streptozotocin (55 mg/kg body wt ip)-induced sustained hyperglycemia and compared with controls. Solid gastric emptying (GE) studies were performed in all the groups. Changes in gastric antrum neuronal nitric oxide synthase (nNOS) mRNA and protein levels were analyzed by real-time PCR and Western immunoblotting, respectively. nNOS dimerization studies were performed using low-temperature SDS-PAGE. In vitro nitricergic relaxation (area under curve/mg tissue wt) was studied after the application of electric field stimulation in an organ bath. Changes in intragastric pressure (mmHg s) in freely moving rats in the presence or absence of Nω-nitro-l-arginine methyl ester (nitric oxide synthase inhibitor) were also higher (P < 0.05). Diabetic females, but not males, showed significant (P < 0.05) impairment in gastric relaxation compared with age-matched normal control rats. The active dimeric form and dimer-to-monomer ratio of nNOS were also higher in healthy females compared with male rats (P < 0.05). Diabetic females, but not males, showed significant (P < 0.05) impairment in both gastric nNOS dimerization and nitricergic relaxation, accompanied by an increase in intragastric pressure. Our data provide evidence that females may have a greater dependency on the nitricergic mechanisms in health. Furthermore, diabetes seems to affect the nitricergic system to a greater extent in females than in males. Together, these changes may account for the greater vulnerability of females to diabetic gastric dysfunction.

solid gastric emptying; nitricergic relaxation; intragastric pressure

GASTRIC DYSMOTILITY OR GASTROPATHY occurs in 20–55% of patients with Type 1 (insulin dependent) and up to 30% of patients with Type 2 diabetes (non-insulin dependent) (37). Symptoms of diabetic gastropathy can range from mild dyspepsia to recurrent vomiting and abdominal pain and are often associated with delayed or accelerated gastric emptying. Fundic tone abnormalities and/or poor antroduodenal coordination has been reported in diabetic patients (28). Although the pathogenesis of diabetic gastropathy is not completely understood, it appears to be much more common (up to four times) in women (46, 22). Such a sex bias is also seen in the idiopathic variety of gastric dysfunction, reinforcing the concept of women being more susceptible to this condition. It is not clear, however, whether this phenomenon suggests a unique biological vulnerability in females or simply an exaggeration of preexisting differences in gastric motility. Thus gastric antroduodenal motility is slower even in healthy women, perhaps owing to hormonal influence (2). Premenopausal women, women during labor, and postmenopausal women receiving hormone replacement therapy develop reversible gastrointestinal complications including delayed gastric emptying for both solids and liquids compared with age-matched men (3, 4, 27). Moreover, studies using male and female rats indicate that estradiol-17β, either alone or in combination with progesterone, may cause delayed gastric emptying (16).

Gastric motility is regulated in large part by neurons of the enteric nervous system located in the muscle wall (53). These neurons are either excitatory (releasing acetylcholine) or inhibitory [releasing nitric oxide (NO) and vasoactive intestinal peptide]. NO is the principal nonadrenergic noncholinergic (NANC) inhibitory neurotransmitter in the gastrointestinal tract and is produced by neuronal NO synthase (nNOS), expressed in inhibitory enteric neurons (48). NO activates soluble guanylate cyclase, producing an increase in the intracellular cGMP leading to muscle relaxation (14, 48). Nitricergic signaling (from NO-releasing neurons) plays a critical role in the control of gastric accommodation and pyloric relaxation in response to a meal. The importance of NO in gastric function was established by the findings of pyloric hypertrophy and gastric dilation in mice with a targeted genomic deletion of nNOS (nNOS−/− mice) (19, 32). Vagal modulation of enteric neural function (both inhibitory and excitatory) also plays an important role in gastric physiology and is predominantly cholinergic in character (29).

Unlike two other NO synthase (NOS) enzymes (endothelial and inducible NOS), both full-length and NH2-terminally truncated forms of nNOS exist because of alternative splicing of 5′ regions of the nNOS gene in both humans and rodents (36, 41, 42). The predominant form of nNOS in the enteric nerves of the stomach is nNOSα, which contains a PDZ/GLGF motif encoded by exon 2 in the NH2-terminal region (41). The

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PDZ/GLGF domain interacts with various proteins and determines nNOSα subcellular localization and function (9, 47). In contrast, the NH2-terminally truncated nNOSβ and nNOSγ lack the PDZ/GLGF motif for protein-protein interaction and possible membrane association. nNOS\(^{−/−}\) mice lacking exon 2 and subsequent membrane-associated full-length nNOSα exhibit delayed gastric emptying of solids and liquids (19, 32). The catalytic activity of NOS depends on dimerization of two NOS poly peptides (25). Dimerization results in the creation of high-affinity binding sites for (6R)-tetrahydrobiopterin (BH4) and arginine in the oxygenase domain and enables electron transfer between flavin and heme groups (7, 17). Enzymatic uncoupling of NOS due to lack of BH4 may in part account for reduced NO production and increased oxidative stress factors such as superoxide (26, 51). The importance of dimerization for the catalytic activity of endothelial NOS and its disruption in diabetes has been reported by several independent investigators (13, 33). However, to the best of our knowledge, there have been no previous in vivo studies on gastrointestinal nNOS dimerization and function.

Although delayed or accelerated gastric emptying has long been taken as a hallmark of diabetes, in recent reports most experts concur that this correlates poorly if at all with clinical symptoms (37, 38). Nitrergic relaxation in gastric muscle preparations obtained from diabetic biobreeding/Worcester (BB/W; an animal model of human Type 1 diabetes) rats is significantly impaired, along with a decrease in nNOS-immunoreactive cells in the gastric myenteric plexus and mRNA expression (49). Similar changes have also been shown in obese and streptozotocin (STZ)-induced diabetic mice and rats (21, 52). Generally, these changes in phenotypic expression have not been accompanied by neuronal loss and, indeed, have been shown to be reversible by insulin treatment (14, 52). It is noteworthy, however, that these studies have all been performed in male animals, despite the knowledge that both nitrergic tissue relaxation and nNOS in gastric fundus can be enhanced by estrogen treatment in female rats (45). Since irregular antral motility may be an important factor responsible for altered gastric emptying in patients, we focused on possible disturbances in nitrergic regulation of gastric motility in the diabetic state (18, 50). The results of our study suggest that nNOS expression, dimerization, and function are sex dependent and state (18, 50). The results of our study suggest that nNOS

fasting male and female diabetic as well as control rats at the time the experiment was performed (12 wk after STZ). Diabetes was confirmed if blood glucose levels ranged from 300–400 mg/dl in both male and female rats. No differences in blood glucose levels were noticed between male and female rats 12 wk after the induction of diabetes with STZ. Age-matched control male and female rats showed blood glucose levels between 80–95 mg/dl. Both control and diabetic rats were selected during the diestrous stage of the estrous cycle. As reported earlier, we noticed that 60–70% of diabetic rats show a persistent diestrous stage of the estrous cycle (data not shown) (8, 24).

Solid gastric emptying studies. Solid gastric emptying studies were performed in healthy male and female rats. Similarly, gastric emptying studies were performed in both male and female rats 8 wk after diabetes induction. The gastric emptying rate of a solid meal was measured as reported previously (31). Groups of healthy and diabetic rats from both sexes were fasted for 20–24 h. A known amount of solid meal with free access to water was supplied for a 3-h period. Food and water were then removed, and the gastric emptying rate of the ingested meal was assessed 4 h later. Following euthanization, the abdominal cavity was opened, the pylorus and cardia were clamped, and the stomach was removed. The amount of food contained in the stomach and solid food ingested by the animals was calculated. The rate of gastric emptying during the 4-h experimental time was calculated according to the following equation: gastric emptying (% in 4 h) = (1 – gastric content/food intake) × 100.

Organ bath studies. For studies on NANC activity, the gastrointestinal tract from the lower esophageal sphincter to the distal duodenum was removed from the body cavity and placed in Krebs bicarbonate solution gassed with a mixture of 95% O2 and 5% CO2. The Krebs (pH 7.4) buffer contains (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO3, 1.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 11.5 glucose. Circular gastric antrum muscle strips were mounted between two L-shaped tissue hooks in 5-ml water-jacketed organ baths containing Krebs buffer at 37°C and continuously bubbled with 95% O2–5% CO2 (Radnoti Glass Technology, Monrovia, CA). Tension for each muscle strip was monitored with an isometric force transducer and analyzed by a digital recording system (Biopac Systems, Santa Barbara, CA). Tension was increased progressively, and contractile responses to potassium chloride (60 mM) were analyzed in Krebs bicarbonate solution measured in various resting conditions (45, 52). Gastric antrum neuromuscular strips were preincubated for 30 min with atropine (10 μM), phenolamine (10 μM), and propranolol (10 μM) to block cholinergic and adrenergic mediated responses. Tone was raised 60–80% of maximal contraction produced by 60 mM KCl by addition of 5-hydroxytryptamine (5-HT) to a final concentration of 100 μM. Specimens demonstrating sustained tonic contractions with 5-HT stimulation were used for the experiments. Nitrergic relaxations were induced for 60 s after contraction with 5-HT by electric field stimulation (EFS; 90 V, 2 Hz, 1-ms pulse for a duration of 1 min, as indicated) (Grass stimulator model SD9, Grass Instruments, AstroMed, West Warwick, RI). The NO dependence of nitrergic relaxations was confirmed by preincubation with N^N-nitro-L-arginine methyl ester (L-NAME, 100 μM; 30 min). All drugs used in this study were purchased from Sigma Chemical. To confirm the role of neuronal depolarization in evoking NANC relaxations, gastric tissues were preincubated for 30 min in the presence of TTX (1 μM; Calbiochem). At the end of each experimental protocol, the muscle strip was removed, 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 (AUC\(_{\text{EFS}}\)) for 1 min and the baseline for 1 min (AUC\(_{\text{B}}\)) according to the formula (AUC\(_{\text{EFS}}\) – AUC\(_{\text{B}}\))/weight of tissue (mg) = AUC/mg of tissue (34).

Ambulatory telemetric studies. Gastric contractility in control and diabetic rats was measured by using an ambulatory telemetric experimental apparatus previously reported for quantifying uterine contractility (10, 11).

MATERIALS AND METHODS

Experimental rats and induction of diabetes mellitus with STZ. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male and female nonpregnant rats (7 wk old) were purchased from Harlan Sprague Dawley (Houston, TX). After arrival at the animal care facility, all rats were maintained in the colony room with a 12:12-h fixed light-dark photoperiod. Animals were allowed free access to water and rodent chow.

Diabetes was induced in groups of male (n = 10) and female (n = 10) rats with a single STZ injection of 55 mg/kg body wt ip (Sigma Chemical, St. Louis, MO) prepared in 5 mM citrate buffer (vehicle), pH 4.0 (30). Control groups were injected with vehicle only. Blood glucose levels from overnight fasting animals were obtained 48 h after STZ to confirm that diabetes was induced in treated animals. Similarly, blood glucose levels were also obtained from overnight
Groups (n = 3–4) of diabetic and control rats were anesthetized with ketamine (45 mg/kg body wt; Fort Dodge Laboratory, Fort Dodge, IA) and xylazine (5 mg/kg body wt; Burns Veterinary Supply, New York, NY). A pressure telemetry transducer/transmitter (CSO-PXT model, Data Sciences, Arden Hills, MN) was implanted into the abdomen of each animal. The transducer pressure catheter was introduced into the gastric antrum cavity 5 mm proximal to the pylorus to record intragastric pressure (IGP, mmHg).

After the baseline IGP was recorded, L-NAME (200 mg per kg body wt; Sigma, St. Louis, MO), an inhibitor of NO synthase, was injected intraperitoneally to reduce intragastric pressure (IGP, mmHg) for 4 days. All subsequent studies on these animals were performed 1 wk after surgery in overnight fasted rats that were awake and in a free-moving state. Pressure recordings were performed at least 2–3 h between 9:00 AM and 12:00 noon throughout the studies.

Data were transmitted by telemetry (RLA 1020 telemetry receivers, Data Sciences), multiplexed (BCM consolidation matrix, Data Sciences), and sent to an adapter where the signal was then demultiplexed, sampled at 1,024 Hz, and converted to analog (UA-10 universal adapter, Data Sciences). This output was band-pass filtered and amplified. The final sampling rate was 10 Hz (10, 11). The information was then fed to a recording system (MacLab 16/s, AD Instruments; Castle Hill, Australia) and stored for later analysis.

For each animal’s data analysis, pressure recordings for at least 10 consecutive contraction events were averaged at each time point. A contraction event was defined as a rapidly occurring increase in IGP of 5 mmHg or more, such that the increased IGP persisted for 5 s or longer (Fig. 1). The pressure integral (or area under the pressure curve) was found for each animal at each time point investigated.

**Real-time RT-PCR.** Total tissue RNA was isolated from the rat gastric antrum neuromuscular tissues by a single-step guanidine thiocyanate method using the reagent Trizol (BRL, Gaithersburg, MD). The quality of RNA was determined by NanoDrop (NanoDrop Technologies), and the quantity was estimated by an Agilent 2100 bioanalyzer (Agilent Technologies, Houston, TX). One microgram of total RNA was denatured at 65°C for 5 min and cDNA synthesis was then performed at 42°C for 1 h by using Superscript reverse transcriptase (BRL). An aliquot of generated cDNA was amplified with a pair of primers (accession number NM_052799) for nNOS (forward 2782–2780, 5'-ACGGACCCCGACGCTCAAGA3', reverse 2856–2837, 5'-CGAGGCGGAAACTGAGAAC-3') and probe [2810–2835, 5'- (FAM)-AAATGTGGGACACTGGCGAATCG-TAMARA]. Quantitative RT-PCR amplification was performed by using two-step TaqMan Universal PCR master mix and the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 repeats of 95°C for 0.15 min and 60°C for 1 min. Relative amounts of mRNA were normalized by 18S and calculated threshold cycle numbers (CT), i.e., 2^-ΔΔCT, according to the manufacturer’s suggestion (Applied Biosystems). All studies were performed in the Molecular Genomics Core Laboratory, The University of Texas Medical Branch, Galveston, TX.

**Western blot analysis.** We examined nNOS protein expression and dimerization using COOH- and NH2-terminal antibodies, respectively. COOH-terminal antibody detects all (total) forms of nNOS (α, β, and γ) whereas NH2-terminal antibody detects only the full-length nNOS protein (41). Under normal conditions, both dimers and monomers of nNOS protein are intensified at 155 kDa. However, low-temperature SDS-PAGE of nonboiled sample homogenates separates NOS dimers (310 kDa) and monomers (155 kDa) (23).

Equal amounts of total protein (40 μg each; Pierce Kit, Rockford, IL) from each preparation were resolved on a 6% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and primed with primary COOH-terminal nNOS monoclonal antibody, respectively. COOH-terminal antibody detects all (total) forms of nNOS (α, β, and γ) whereas NH2-terminal antibody detects only the full-length nNOS protein (41). Under normal conditions, both dimers and monomers of nNOS protein are intensified at 155 kDa. However, low-temperature SDS-PAGE of nonboiled sample homogenates separates NOS dimers (310 kDa) and monomers (155 kDa) (23).

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**Statistical analysis.** Results are expressed as means ± SE obtained from four to seven animals for solid gastric emptying and organ bath studies and three to four samples for both IGP and nNOS expression studies. Data were analyzed for statistical differences with Student’s t-test or two-way ANOVA followed by the Bonferroni t-test to verify the differences between individual groups. P < 0.05 was considered significant (n = 3–6).

**RESULTS**

Sex-dependent gastric emptying rate in control and diabetic rats. Figure 2 shows differences in solid gastric emptying rate in healthy and diabetic male and female rats. Healthy females during the diestrous stage of their estrous cycle (when circulatory estrogens are beginning to be elevated) exhibited a trend toward a delay in gastric emptying for solids compared with age-matched male rats. However, this was not statistically

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**Fig. 1.** Intragastric pressure (IGP) contractions were observed using implanted telemetric IGP devices. Contractions were defined as rapidly increasing pressure events, 5 mmHg or more in amplitude, with a duration of 5 s or more. These pressure curves were isolated and an integral from baseline was calculated. Ten such consecutive contractions were evaluated in this way to obtain a mean IGP for each animal for each time point. Means and SE of the IGP were then found for each group of animals at each time point.
Diabetes impaired gastric nNOS dimer function

Sex-dependent nitrergic relaxation in control and diabetic rats. Circular muscle strips from gastric antrum from both control and diabetic rats showed nitrergic relaxation in response to EFS using a frequency of 2 Hz. This relaxation was significantly antagonized by preincubation with the NOS inhibitor L-NAME (100 μM) and completely abolished in the presence of TTX (data not shown), confirming its nitrergic nature and neuronal source, respectively (Fig. 3).

Tissues obtained from female control showed substantially greater nitrergic relaxation compared with the male control group (P < 0.05). Furthermore, nitrergic relaxation was significantly impaired (P < 0.05) in female diabetic rats but not in male diabetic controls, compared with their respective control groups (Fig. 3).

Sex-dependent IGP in control and diabetic rats. Intraluminal antral pressure was measured in age-matched male and female controls (Fig. 4A) and 12 wk after induction of diabetes in controls and in L-NAME-treated animals (Fig. 4B). As shown in Figs. 4C and 3D, the female control group showed a significant (P < 0.05) reduction in IGP (mmHg·s) compared with male controls. L-NAME infusion attenuated the lowered antral pressure in female controls, implicating a role for the nitrergic system in this phenomenon. Diabetes caused a significant (P < 0.05) IGP elevation in female diabetic rats compared with female controls (Fig. 4D), and this did not change after L-NAME infusion, suggesting loss of nitrergic tone. By contrast, IGP was reduced in male diabetic rats compared with male controls (Fig. 4C), an effect that was at least partly reversed by L-NAME administration (Fig. 4C), suggesting relative preservation of nitrergic tone.

Sex-dependent nNOS expression in gastric tissues in control and diabetic rats. Compared with their male counterparts, female controls had higher nNOS mRNA (Fig. 5A), but not total protein levels (Fig. 5B). Diabetes did not appear to affect the expression of either mRNA or protein in females. However, both mRNA and total protein expression was significantly (P < 0.05) elevated in gastric antrum obtained from male diabetic rats compared with their respective controls (Fig. 5).

Dimerization of nNOSα in gastric antrum. We next set out to determine changes in dimerization of nNOSα in the various experimental states. We examined this by low-temperature SDS-PAGE so that the SDS-resistant dimeric form of nNOSα could be measured. This assay is not a measure of the absolute dimeric content under native conditions, but it is a measure of the amount of stable dimer that is not dissociated by SDS and thus somewhat underestimates the total dimeric content. Nonetheless, it is a convenient and reliable measure for studying relative changes in the dimeric state of nNOS. We found that the ratio of nNOSα dimers to monomers was significantly increased (P < 0.05) in female control rats compared with male controls in antrum (Fig. 6). Diabetes resulted in significant (P < 0.05) decreases in nNOSα dimerization in female antral tissue but not in males, compared with their respective controls (Fig. 6).

Figure 7 shows the changes in gastric emptying (A), gastric antrum nitrergic relaxation (B) and nNOSα protein dimer.
levels (C) in diabetic male and diabetic females after normalization to their own controls. As can be seen, diabetes significantly (P < 0.05) reduced gastric emptying, nitrergic relaxation, and nNOS protein dimer levels in females compared with male rats (Fig. 7). The decline in gastric antrum nNOS dimer levels correlated with changes in gastric antrum nitrergic relaxation and solid gastric emptying in diabetic female rats (Fig. 7).

**DISCUSSION**

Abnormal gastrointestinal motility in diabetes mellitus is likely multifactorial in origin, reflecting disturbances in enteric and vagal neural activity as well as interstitial cells of Cajal and smooth muscle function (20, 28, 33, 38). Of these, enteric neuropathy may be particularly important (5, 6, 48). Several studies of animal models of diabetes have convincingly shown disturbances in enteric nerves, particularly involving nitrergic nerves (14, 49, 52). Since these studies have used male rats exclusively, it has been difficult to correlate these findings with the clinical finding that 80% of all gastroparetic patients are female. It is possible that the pathogenetic mechanisms of diabetic gastric dysmotility are common to both men and women but the latter are disproportionately symptomatic because the motility of their stomachs is slower to begin with. On the other hand, it is also possible that there are fundamental sex-specific differences between how the gastric neuromuscular apparatus is affected by diabetes. The results of our study suggest that the latter explanation may be more likely.

In this study, we have demonstrated sex differences in solid gastric emptying, antral nNOS expression, nitrergic relaxation, IGP, and nNOS protein dimerization in both diabetic and nondiabetic rats. Compared with control male rats, healthy females during the diestrous stage of the estrous cycle showed delayed gastric emptying (Fig. 2). However, this was not statistically significant. Previous studies have demonstrated that estradiol-17β administration delays gastric emptying for liquids in both male and female rats (16). Thus, more studies are warranted to address whether solid gastric emptying rate is different during various stages of the estrous cycle when estrogen levels are higher than in the diestrous phase. However, nitrergic relaxation of the antrum is clearly greater in healthy diestrous females (Fig. 3) compared with males, and
this correlates with a corresponding increase in both total nNOS protein expression (Fig. 5) and nNOSα dimerization (Fig. 6). Our studies further demonstrate that IGP is significantly lower in females compared with male rats (Fig. 4, C and D). This is in keeping with human studies showing that gastric antral contractility and gastric emptying is slower in young women compared with men whereas circulatory estrogen levels are elevated (27). Their method for measuring antral contractility was not telemetric, but since the study was performed in humans, the externalized catheters posed little problem for implementation insofar as the integrity of the data is concerned. In animals this is not the case, where with externalized catheters, either the animal must be restrained (introducing undue stress) to prevent destruction of the measurement apparatus or the data must be acquired while the animals are

Fig. 5. Sex- and diabetes-related changes in the expression of neuronal nitric oxide synthase (nNOS) in the gastric distal antrum of male control, female control, male diabetic, and female diabetic rats. nNOS mRNA (A) and protein (B) levels were measured from gastric antrum by quantitative RT-PCR and Western blot analysis, respectively. Diabetest was induced with a single injection of STZ (55 mg/kg body wt ip) and all studies were performed after 12 wk of diabetes induction. The control group received vehicle (citrate buffer). A: nNOS and 18S mRNA expressions were measured by real-time RT-PCR. Relative abundance of nNOS mRNA normalized by 18S were loaded on each lane and detected with polyclonal antibody specific for the exon 2 encoded NH₂-terminal domain of nNOSα. Top: representative immunoblot of gastric distal antrum homogenates (n = 3–4) showing the ratio of nNOSα dimers (310 kDa) to monomers (155 kDa). Bottom: densitometric analysis followed by a ratio of nNOSα dimerization to γ-tubulin is calculated. Bars represent means ± SE. Significant differences from control vs. diabetes are noted. *P < 0.05 for male diabetic compared with male control and #P < 0.05 for female diabetic compared with female control.

Fig. 6. Sex- and diabetes-related changes in the expression of neuronal nitric oxide-α dimerization (nNOSα) in the gastric distal antrum of male control, female control, male diabetic, and female diabetic rats. Equal amounts of protein (40 μg) were loaded on each lane and detected with polyclonal antibody specific for the exon 2 encoded NH₂-terminal domain of nNOSα. Top: representative immunoblot of gastric distal antrum homogenates (n = 3–4) showing the ratio of nNOSα dimers (310 kDa) to monomers (155 kDa). Bottom: densitometric analysis followed by a ratio of nNOSα dimerization to γ-tubulin is calculated. Bars represent means ± SE. Significant differences from control vs. diabetes are noted. *P < 0.05 for female control compared with male control and #P < 0.05 for female diabetic compared with female control.
unconscious. Both of these alternatives may lead to spurious data. By using a telemetric device in conscious, unrestrained animals, we obtained the most accurate measurement for intraluminal pressure. In our study, L-NAME infusion increased IGP significantly in female but not in male rats, suggesting that nonnitrergic mechanisms may be more important in the latter (Fig. 4, C and D). Thus it appears that females rely on nitrergic control of gastric motility to a greater extent than males and hence may be more vulnerable to alterations of this system induced by diabetes.

We next turned our attention to changes in these parameters after induction of diabetes. The rate of gastric emptying may vary depending upon the type of method employed, i.e., liquids vs. solids; type, duration, and severity of diabetes. Human studies have demonstrated that diabetic women have more delayed gastric emptying compared with diabetic men (22, 46). In the present study, we have demonstrated that solid gastric emptying (A), nitrergic relaxation (B), and nNOS protein dimer levels (C) in diabetic female gastric tissues compared with diabetic male rats. The changes in the above parameters were normalized with their respective control group. Data were presented as % change over control. *P < 0.05 (ANOVA).

Fig. 7. Sex-dependent changes in solid gastric emptying (A), gastric antrum nitrergic relaxation (B) and gastric antrum nNOS dimer levels (C) in healthy and diabetic rats. Diabetes was induced with a single injection of STZ (55 mg/kg body wt ip). The control group received vehicle (citrate buffer). Diabetes reduced solid gastric emptying (A), nitrergic relaxation (B), and nNOS protein dimer levels (C) in diabetic female gastric tissues compared with diabetic male rats. The changes in the above parameters were normalized with their respective control group. Data were presented as % change over control. *P < 0.05 (ANOVA).
In diabetic gastric dysfunction, antral motility and the coordination of pressures between the antrum and duodenum are diminished (28, 46, 53). Antral hypomotility has been recorded with intraluminal pressure transducers in patients with diabetes mellitus. In this study, we have shown that nitrergic relaxation (Fig. 3) is significantly reduced with concomitant increases in IGP in female diabetic rats (Fig. 4D). Furthermore, nNOSα dimerization (Fig. 6) but not total (Fig. 5) nNOS expression is significantly reduced in these animals. After normalization with their own control group, diabetic females exhibited significant reduction in solid gastric emptying (Fig. 7A), gastric antrum nitrergic relaxation (Fig. 7B), and nNOSα protein dimer levels (Fig. 7C) compared with male rats. These data suggest that a decline in nNOSα dimerization correlates with changes in nitrergic relaxation and gastric emptying and may explain why females have a greater tendency to gastroparesis.

In this study, we also demonstrate an increase in gastric antrum nNOS expression in the male diabetic group. Although our results are similar to those reported by Adeghate et al. (1), they are in contrast to most of the previous literature (all of which deals exclusively with male rats) reporting a decrease in gastric nNOS expression in diabetic rats (48, 49, 52). We are unable to satisfactorily explain this discrepancy but do note that we have utilized a lower dose of STZ to induce diabetes compared with others (15, 49). Furthermore, by contrast to female diabetic rats, we were unable to show a significant reduction in nNOSα dimerization or nitrergic relaxation in male diabetic animals. Interestingly, diabetes resulted in reduced IGP in males (compared with an increase in females), again suggesting a potentially important sex difference in the control of gastric motility (Fig. 4, C and D). The mechanism underlying the reduction in IGP in diabetic males remains unknown but may be due to other factors such as impairment of interstitial cells of Cajal dysfunction and antral electrical pacemaking and neurotransmitter coupling. More studies are needed to understand the biological basis of this sex effect.

The mechanisms regulating changes in nNOS dimerization and nitrergic function in female diabetic animals are not addressed in the present investigation but may be related to alterations in female hormonal control. Circulatory estradiol levels are lower in diabetic women and rats (30, 39). Estradiol-17β increases both nNOS expression and GTP cyclohydrolase 1 expression (43, 44). The latter is a rate-limiting enzyme for the production of BH4, which is required for dimerization of nNOS (12). Therefore, we suggest that the decrease in estradiol in diabetic female gastric tissues results in a reduced BH4 synthesis with adverse effects on nNOS activity, thus leading to increased gastric dysmotility. Studies are in progress to address this issue.

In summary, this study demonstrates significant sex differences in gastric emptying, gastric antral nitrergic function, and nNOS expression and dimerization in both health and disease. Nitrergic relaxation is more pronounced in healthy females, accounted for, perhaps, by an increased expression of the active dimeric form of nNOSα. On the other hand, chronic hyperglycemia causes a greater reduction in active forms of nNOS in females associated with significantly greater impairment of nitrergic relaxation. This study also illustrates for the first time the importance of nNOSα dimerization in gastric physiology. These findings may provide a biological explanation for the greater vulnerability of females to develop diabetic gastric dysmotility problems.

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